Identification of genes required for cold acclimation using next generation sequencing of sensitive to freezing mutants

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Identification of genes required for cold acclimation using next generation sequencing of *sensitive to freezing* mutants

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ABSTRACT

Wild type Arabidopsis plants are able to survive temperatures below 0°C, providing they have been subjected to low positive temperatures for several days prior to freezing. This brief period of low temperature is known as cold acclimation and results in the activation of a number of pathways that bring about physical and biochemical changes necessary to withstand freezing stress. However, when the sensitive to freezing (*sfr*) mutants are subjected to temperatures that would promote acclimation in wild type plants, they fail to become more freezing-tolerant. This suggests that the mutation lies in a gene intrinsic to the acclimation process.

The sensitive to freezing mutants were discovered as part of an ethyl methanesulfonate (EMS) chemical mutagenesis screen, which induces single nucleotide polymorphisms (SNPs) into the genome. As a result, for each mutant the deficiency in freezing tolerance is caused by a SNP in an unknown gene. *sfr4*, *sfr5*, *sfr8* and *sfr9* were the focus of this investigation. Mapping intervals had been previously determined for each of the four *sfr* mutants using classical recombination-based techniques, giving a specific region of the genome in which to search for the SNP responsible for freezing sensitivity.

Full Illumina genome sequences were produced for *sfr4*, *sfr5*, *sfr8* and *sfr9*, and these were mapped against the TAIR 10 Arabidopsis genome. Using the Integrative Genomics Viewer software, the mapping interval was scanned and all SNPs of interest were recorded. Additionally, a command line-based method of identifying SNPs was also applied to the sequencing data by collaborators at the University of Liverpool. SNPs found by both methods were verified to exist in the DNA of the mutant they corresponded to via direct DNA sequencing. This provided a number of candidate genes for each *sfr* mutant line. T-DNA insertional mutants were obtained for each of the genes in question, and these were genotyped via PCR. Homozygotes were tested for freezing tolerance, comparing them to the respective *sfr* mutant to the knockouts/knockdowns in each potential gene candidate.

Additional phenotypic data was collected for each of the *sfr* mutants, as they had not received much work prior to the investigation. At the end of this investigation, several candidates remained as potential causes of freezing tolerance for *sfr5*, *sfr8* and *sfr9*; however no candidates were isolated for *sfr4.*

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1. INTRODUCTION

As sessile organisms, plants must react to the environmental conditions to which they are subjected, and one condition that can vary greatly is ambient temperature. While drought is commonly perceived as the greatest limiter of crop production, it is important to note that approximately two thirds of the Earth's land mass experiences temperatures below 0˚C at some point during every year, and half is subjected to temperatures that drop lower than –20°C (Larcher 1994). When even chilling temperatures are able to cause damage to crops (Hetherington *et al.* 1989), freezing temperatures will seriously limit the area in which certain plants can be cultivated.

Tolerance of frost is extremely important to many crop species such as *Triticum aestivum* (winter wheat), however in recent years climate change has resulted in frosts in areas of the world where this would not normally be expected, causing damage to crops that would not usually experience such low temperatures (Gu *et al.* 2008, Warmund *et al.* 2008). Understanding how plants sense and respond to cold temperatures will be crucial for the production of plants engineered to tolerate freezing temperatures.

1.1. Chilling and freezing tolerance

The degree to which different plant species can tolerate low temperatures varies greatly. Some plants are unable to tolerate low positive temperatures, and temperatures between 10°C and 12°C can cause damage to them (Levitt 1980). These plants are known as chilling sensitive and are usually either native tropical plants, or annuals, such as rice and maize, which undergo only one breeding season. Plants that can withstand low positive

temperatures are known as chilling resistant, and can be sub-divided into two further categories; freezing sensitive and freezing tolerant. Freezing sensitive plants die when subjected to temperatures below 0°C regardless of any other conditions. Freezing tolerant species are able to survive sub-zero temperatures; some species have constitutively active defences whereas others have defences that first need to be activated. This activation process is known as cold acclimation. In *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), it has been suggested that over 1000 genes are induced by cold (Kilian *et al.* 2007), and of these more than 170 encode transcription factors, however some estimates are much higher (Hannah *et al.* 2005). Some transcription factors are known to control a wide range of genes; the most prominent example being the C-repeat binding factor (CBF) transcription factors (Stockinger *et al.* 1997, Jaglo-Ottosen *et al.* 1998, Medina *et al.* 1999), which mediate the expression of a large number of genes that constitute the inducible freezing tolerance of plants.

1.2. Freezing damage

There are various ways in which plants are damaged when exposed to freezing temperatures. Woody species are particularly prone to embolisms in the xylem, in which gas bubbles are forced out of solution when freezing occurs (Sperry and Sullivan 1992). These gas bubbles break the continuous water column (an event known as cavitation), which disrupts the flow of water up a plant (Van den Honert 1948). Cavitation can persist for months and can both limit growth and result in shoot dieback (Sperry *et al.* 1993, Tyree and Cochard 1996). It has also been shown that ice formation within plant tissue can cause cellular damage which can lead to cell rupture (Ristic and Ashworth 1993, Uemura *et al.* 1995). However, it has been recognised for over 100 years, that injury to the plant cell

membranes has the most severe effect on plant survival of freezing, and that the plasma membranes are most damaged by cellular dehydration (Levitt 1980).

The intercellular fluid of plant cells has a much lower solute concentration than the intracellular fluid, and as a result, freezes at a higher temperature. Due to this formation of ice outside the cells, the water potential of the extracellular space is reduced, and this results in water being drawn out from the cytosol, down the concentration gradient (Uemura and Steponkus 1989). Due to these differences in water potential across the plasma membrane, damage can occur. It is this dehydration damage which draws parallels between freezing and drought stress, and the positive responses of plants to mediate drought damage use similar signalling components to those of freezing (Nakashima *et al.* 2000).

Freezing experiments carried out on protoplasts have demonstrated that changes occur to the organisation of the lipid bi-layer upon freezing (Webb and Steponkus 1993), however, it must be noted that whilst it is likely that protoplasts do portray an accurate representation of what is observed in nature, it has not yet been verified that this is what occurs in whole cells. In protoplasts the cell wall is absent, which presents an obvious limitation; it is quite likely that the cell wall will provide the plasma membrane with protection from ice crystal formation, and without the cell wall the damage protoplasts experience may be more severe (Minami *et al.* 2009).

Due to the loss of water from plant cells when they are frozen, vesicles of the plasma membrane bud off, disrupting the structure. During the thawing process water moves into

the cell, but due to the newly reduced size of the plasma membrane the cell bursts; this is known as 'Expansion-induced lysis' (Webb *et al.* 1994, Uemura and Steponkus 2003)**.** This type of damage is only seen in non-acclimated protoplasts. The electrolytes that leak out from these membrane lesions can be measured quantitatively, and can consequently be used to provide data that demonstrate the level of damage caused to a plant by freezing.

Another type of damage that is seen in response to freezing in non-acclimated protoplasts is the 'Hexagonal II phase' membrane lesion. This occurs between approximately -2°C and -4°C in Arabidopsis (Uemura *et al.* 1995) and is characterised by plasma membrane destabilisation, causing membrane lipids to aggregate together forming tubes within the membrane, known as the 'Hexagonal II Phase'. This occurs when two membranes are close together, such as when the plasma membrane and chloroplast membrane are in close proximity (Uemura *et al.* 1995), which is likely to occur if the cell has lost a large proportion of its liquid. Both 'Expansion-induced lysis' and 'Hexagonal II phase' membrane lesions are potentially eliminated by acclimation (Webb *et al.* 1994, Uemura *et al.* 1995).

There is one type of lesion, similar to 'Hexagonal II Phase', which is found exclusively in acclimated protoplasts; the 'Fracture Jump' lesion. While 'Hexagonal II Phase' lesions are never seen in acclimated protoplasts, these lesions are (Uemura *et al.* 1995). The 'Fracture Jump' lesion occurs when protoplasts (taken from acclimated plants) are frozen to -25°C, and appears as a localised deviation from the fracture plane of the plasma membrane (Webb and Steponkus 1993). Despite the potential differences that may occur between the response of living plant cells to those of protoplasts, it is known that the membrane is the

predominant site of injury in freezing, and protecting the membrane from destabilisation is key.

1.3. How membranes are protected

The plasma membrane (Gordon-Kamm and Steponkus 1984, Uemura *et al.* 1995), thylakoid membranes (Hincha *et al.* 1984), and the chloroplast envelope (Krause and Santarius 1975) are all severely damaged by freezing. As a result, many of the changes that occur during cold acclimation involve protecting cellular membranes, particularly in counteracting changes in cell and organelle volume caused by the desiccation that occurs upon freezing.

In some aspects the membrane is able to protect itself; alterations in the lipid composition of the membrane has been linked to an increase in freezing tolerance (Uemura and Steponkus 1994, Uemura *et al.* 1995). The fatty acid chains that are incorporated into the plasma membrane can be of varying lengths and can display varying degrees of saturation. Shorter chain lipids are less viscous and as result reduce rigidification of the membrane on exposure to cold. A higher number of double bonds in a fatty acid chain increase fluidity; these chains are unsaturated with hydrogens and as a result do not tessellate as readily as more saturated chains (which display a lower number of double bonds); as a result a membrane made up predominantly of unsaturated chains requires less heat to remain fluid (Mironov *et al.* 2012). Some of the enzymes involved in making membranes more tolerant to freezing are those that alter the saturation of these chains, such as *FAD2*, which is discussed in detail in section 1.5 (Okuley *et al.* 1994).

It has been shown that after as little as six hours of cold acclimation, increases in freezing tolerance are evident before changes in the plasma membrane structure can be seen (Ristic and Ashworth 1993, Uemura *et al.* 1995, Wanner and Junttila 1999). Storage lipids, specifically long-chain unsaturated triacylglycerides are greatly accumulated in the plasma membrane during cold acclimation, providing another aspect of protection (Degenkolbe *et al.* 2012). The changes that occur within the cellular membranes can be so specific that certain lipids can be used as a marker for increased freezing tolerance. If a plant has a higher proportion of these lipids it will have a higher tolerance to freezing (Degenkolbe *et al.* 2012). In the chloroplast envelope it has been shown that acclimation results in changes in the activity or expression of enzymes that result in altered membrane composition. These enzymes alter the chemical groups attached to membrane lipids, and have the effect of creating lipids that are more amenable to forming a bilayer. This prevents non-bilayer structures such as 'Hexagonal II phase' (section 1.2) from forming, which would result in and a loss of membrane integrity. SFR2 (discussed in detail in section 1.10.1) is one such enzyme (Moellering *et al.* 2010). However, these defences alone do not do enough to keep membranes functional and extra mechanisms must be in place.

As well as membrane reorganisation, there are a number of mechanisms involved in preventing membrane rupture. The accumulation of various compatible solutes, which are metabolically inert compounds with no charge, such as proline, occurs to combat the solute potential caused by freezing in intracellular spaces (Wanner and Junttila 1999, Kaplan *et al.* 2004). The eskimo 1 (*esk1*) mutant, a constitutively active freezing mutant, accumulates proline even at ambient temperatures, 30-fold higher than non-acclimated wild type Arabidopsis (Xin and Browse 1998).

The sensitive to freezing mutant *sfr4* lacks the ability to accumulate sucrose and displays a complete lack of cold acclimation; this lack of sucrose has been found to be causative of the freezing tolerance deficiency (Uemura and Steponkus 2003). There are several theories to explain the mechanism by which sucrose may protect plant cells from freezing damage; one such method could be to prevent the dehydration caused by freezing stress, by keeping water molecules within the cell (Steponkus 1984) thereby potentially promoting membrane stability (Lineberger and Steponkus 1980). It may also act as a cryo-protectant for enzymes (Carpenter *et al.* 1986). It has been shown that sucrose, along with trehalose, a disaccharide, is extremely effective at providing protection for the membranes (Anchordoguy *et al.* 1987). Increased levels of trehalose have been shown to increase drought tolerance in rice, thus highlighting the potential importance of trehalose and other soluble sugars in the protection of cells against the desiccation that occurs during freezing (Garg *et al*. 2002). However, as with the majority of plant defences against freezing, the ability to accumulate soluble sugars alone does not lead to freezing tolerance; the majority *sfr* mutants are able to accumulate sucrose but are completely deficient in freezing tolerance (McKown *et al.* 1996).

With the discovery that several of the original *sfr* mutants, including *sfr4*, showed a deficit in anthocyanin accumulation when subjected to cold acclimation (McKown *et al.* 1996), the role of anthocyanin in freezing tolerance was considered. Cold temperatures have been shown to up-regulate anthocyanin biosynthesis in Arabidopsis (Leyva *et al.* 1995, McKown *et al*. 1996), however no definitive evidence has been produce to suggest that this directly increases freezing tolerance. While anthocyanin itself is not likely to be a key factor in cold acclimation, it has been suggested that the anthocyanin biosynthesis pathway may share

some common regulatory elements with a pathway that is involved in freezing tolerance (McKown *et al.* 1996).

Group 2 late embyrogenesis (LEA) proteins, also known as dehydrins, have been shown to accumulate as part of cold acclimation, and protect plants from damage when freezing occurs (Close 2006). Dehydrins are hydrophilic and thermostable, and it has been suggested that they protect cells from dehydration (like compatible solutes) by adding stability to the plasma membrane. It has been suggested that they essentially act as a surfactant by preventing coagulation of membrane structures (Ismail *et al.* 1999). In Arabidopsis, it has been shown that Low-Temperature-Induced 30 (LTI30) and Cold-Regulated 47 (COR47) are dehydrins that accumulate specifically in response to cold temperature (Nylander *et al.* 2001), suggesting that their roles are not simply specific to the dehydration pathway and that they are likely to play a role in cold tolerance.

Altered gene expression (when measured as changes to levels of mRNA transcript and altered enzyme activity) can be seen within a few hours of cold conditions (Wanner and Junttila 1999), however longer-term changes to safeguard the plant against freezing damage such as decreased water content and structural changes take days or weeks to occur (Uemura *et al.* 1995).

1.4. Cold acclimation

Cold acclimation occurs when a plant has spent a period of time (days or weeks) at low positive temperatures, accepted to be around 5°C. In nature it corresponds to late autumn, allowing plants to prepare for sub-zero winter temperatures. This warning period allows it to activate mechanisms that facilitate the protection of the plant against subsequent freezing temperatures (Guy *et al.* 1987). It is known that as little as 12 hours of acclimation time provides Arabidopsis with some level of tolerance (Jaglo-Ottosen *et al.* 1998), however in most investigations is has been seen that a much longer time is required to provide the plant with fully functioning defences. Seven days has been found to be the minimum time required for complete cold acclimation to occur (Guy *et al.* 1987). Acclimation does not result in permanent protection from freezing; even when freezing tolerance has previously been activated in a plant it is lost after one to two days of exposure to warmer temperatures. This therefore suggests that, for species possessing inducible tolerance, a constitutively active freezing pathway would be too costly to maintain (Wanner and Junttila 1999).

A large number of the genes involved in the early stages of cold acclimation are transcription factors, and the discovery of the CBFs uncovered one of the major regulators of acclimation. The CBF transcription factors that are expressed in response to cold and activate a large number of genes that bring about increases in freezing tolerance (described in section 1.6). The CBFs bring about huge changes in what is being transcribed, as is shown when they are overexpressed (Gilmour *et al.* 2004). An example gene that is clearly induced by cold and the CBF transcription factors, Cold-Regulated 15a (COR15a), has been linked to protecting the chloroplast membrane from damage (Nakayama *et al.* 2007).

However, not all of the changes that occur during acclimation rely on changes in the expression of genes; as previously mentioned there are a number of physical changes that occur in the membranes that alter their structure and composition and negate the water loss that occurs during freezing. Likewise there are metabolic changes, and the accumulation of compatible solutes, soluble sugars and dehydrins to mediate dehydration (Chen and Murata 2002, Klotke *et al*. 2004).

1.5. Cold sensing

The process of cold acclimation relies on the assumption that plants have an ability to sense the low positive temperatures. There is a vast array of literature on the topic of moving between the initial drop in temperature and how the plant responds to this, and a number of pathways have been shown to be required for plants to respond to cold, however it has not been proven that any or all of these are needed for the acclimation process.

Due to the damage that cold temperatures can cause to membranes, it is not unsurprising that the plasma membrane has been theorised to be the first step in the pathway to sensing a temperature drop (Levitt 1980). In wild type Arabidopsis, at ambient temperatures, the cellular membranes are fluid. However, when temperatures decrease, the phospholipids within the membrane re-arrange their structure, to become less saturated, and as a result the membranes become more rigid (Alonso *et al.* 1997). The Arabidopsis fatty acid desaturase mutant (*fad2*) displays membrane rigidification at a higher temperature than the wild type plant, and lacks an enzyme essential for the synthesis of polyunsaturated lipids (Okuley *et al.* 1994). A combination of chemical treatments promoting membrane rigidification and blocking cytoskeletal rearrangement, allows Cold Acclimation-Specific 30

(*CAS30)* transcript accumulation. If membrane rigidifcation is blocked, accumilation of CAS30 is not seen. (Örvar *et al.* 2000).

A rapid calcium influx is seen early in response to cold in Arabidopsis (Knight *et al.* 1996), and calcium has been shown to be an important secondary messenger for the activation of some, if not all, cold up-regulated genes, and has also been shown to be required for cold acclimation (Tahtiharju *et al.* 1997). It has been suggested that cytoskeletal changes may lead to the opening of calcium channels, allowing calcium to move into the cell from extracellular stores. When chemical inhibitors were used to block cytoskeletal rearrangement, this resulted in reduced cold gene expression (Örvar *et al.* 2000). As of yet, these channels have not yet been identified, but it has been suggested that they are mechano-sensitive (Carpaneto *et al.* 2007). It has been suggested that when calcium enters cells it binds to calcium sensors, which allow it to initiate downstream effects (Huang *et al.* 2012). For further information reviews are available on this subject (Harper *et al.* 2004).

One proposed link between the increase in calcium and gene expression is a series of mitogen-activated protein kinases (MAPKs). It is not known if or how the calcium sensors interact with the MAPK cascade, however the Calcium/Calmodulin-Regulated Receptor-Like Kinase CRLK1 (which is bound by calcium/calmodulin to activate its kinase activity) has been shown to interact with MAPK/ERK Kinase Kinase 1 (MEKK1); moreover, the calcium cascade cannot proceed without it (Yang *et al.* 2010). There is also evidence of calcium-dependent protein kinases regulating freezing tolerance in Arabidopsis (Harper *et al.* 2004), which suggests that they may provide the link between calcium elevation and the kinase cascade.

It has been shown in Arabidopsis protoplasts that mitogen-activated protein kinase kinase kinases (MAPKKKs, specifically MEKK1) are induced in response to cold (Teige *et al.* 2004). MEKK1 specifically induces Mitogen-Activated Protein Kinase Kinase 2 (MAPKK2), and under cold conditions its major target is mitogen-activated protein kinase 4 (MAPK4). Phosphorylation of MAPK4 has been proven in leaf tissue. The *mkk2* knockout mutant is unable to cold acclimate, proving that this is a vital stage in the lead-up to freezing tolerance (Teige *et al.* 2004).

1.6. C-repeat binding factors (CBFs)

Three CBF transcription factors have been identified in Arabidopsis as being important for freezing tolerance; these genes, *CBF1, CBF2 and CBF3*, are located in tandem array on chromosome IV (Jaglo-Ottosen *et al.* 1998). Ectopic expression of the CBFs has been shown to induce the cold acclimation process to occur even at ambient temperatures (Stockinger *et al.* 1997, Liu *et al.* 1998). Homologs of the CBFs have been found in *Brassica napus* (Jiang *et al.* 2011), barley (Morran *et al.* 2011), and even chilling-sensitive plants such as rice (Ito *et al.* 2006). It has been suggested that 478 cold-regulated genes are regulated via by the CBFs, and as a result will bring about a number of transcriptome changes in response to cold (Hannah *et al.* 2005).

CBF transcription factors act by binding to the CRT (C-Repeat); a sequence with a core repetition of the bases 5'-CCGAC-3' that is found in the promoter region of *COR* (cold-regulated) genes (Baker *et al.* 1994, Stockinger *et al.* 1997). Experiments in yeast have shown CBF1 is a transcriptional activator of genes containing the CRT; the CBFs themselves

do not contain the CRT, however they are induced by cold. Over-expressing *CBF1* has been shown to increase freezing tolerance by 3.3°C (Jaglo-Ottosen *et al.* 1998).

The CRT motif is also present in the dehydration-responsive element (DRE), which is a nine base repeat of 5'-TACCGACAT-'3. The DRE is found in the promoters of genes that are activated in drought conditions (Yamaguchi-Shinozaki and Shinozaki 1994). The CBF family are also known as the Dehydration-Response-Element-Binding 1 (DREB1) family, and the CBFs/DREB1s are activated in response to cold. Another family of DREB proteins, the DREB2s are activated by high-salinity and drought stresses (Liu *et al.* 1998, Nakashima *et al.* 2000) and these bind to the DRE. It has been suggested that there is cross-talk between DREB1s/CBFs and DREB2s, potentially via CBF4 (Haake *et al.* 2002). CBF4 is an apparent homolog of CBF1, and performs the equivalent role in drought tolerance. This suggests the two pathways may have evolved from the same transcription factors.

It has been shown in Arabidopsis that *CBF* transcription is not up-regulated in response to drought, and is specific to cold temperatures (Jaglo-Ottosen *et al.* 1998). Induction of the *CBF* genes has been seen to occur less than fifteen minutes after exposure to 4°C in Arabidopsis (Jaglo-Ottosen *et al.* 1998), with the induction of target genes of CBF transcription factors occurring two to three hours after transfer to cold conditions. Experiments have shown that the expression of single *COR* genes, such as *COR15a*, do not bring about enhanced freezing tolerance. This suggests that these *CBFs* control a complex regulatory pathway to bring about freezing tolerance. Regulation of the CBFs is obviously controlled as overexpression CBF1 has an adverse effect on the health of the plant; it is dwarfed, has low seed set, and is late flowering (Jaglo-Ottosen *et al.* 1998, Kasuga *et al.*

1999). CBF1 and CBF3 function additively, whereas CBF2 acts differently (Novillo *et al.* 2007). The *cbf2* null mutant results in increased expression of CBF1 and CBF3, and of the genes downstream from them, suggesting the CBF2 itself plays a part in the regulation of the other CBFs. The *cbf2* null mutant shows increased freezing tolerance (Catalá *et al.* 2011). Recent work has shown that CBF2 is subjected to both positive and negative regulation. Five Altered CBF2 Expression (*acex*) mutants which were neither allelic to one another, nor constitutively active, were all seen to have altered expression of the CBFs, with one mutant displaying only reduced CBF2 expression and the remaining four displaying reduced expression of all CBFs (Novillo *et al.* 2012). The mutants which display reduced expression of *CBF1* and *CBF3* when only *CBF2* has been affected supports the idea that they are themselves regulated by CBF2.

1.7. Regulation of the CBFs

Due to the fast induction of *CBF* gene expression in response to cold temperatures, it seemed likely that some factor must be present in cells at ambient temperatures, ready to activate *CBF* transcription. In 1998, Gilmour *et al.* proposed the existence of gene, which they named Inducer of CBF Expression *(ICE) that* was responsible for the activation of the *CBF* genes. Two ICE proteins have been identified, named ICE1 and ICE2 (Chinnusamy *et al.* 2003, Fursova *et al.* 2009). ICE1 was found via the screening of an EMS-mutagenised transgenic population of Arabidopsis plants, expressing a fusion of the firefly luciferase reporter gene to the promoter of *CBF3*, resulting in the photoluminescence of plants in response to cold temperatures; *ice1* mutants were easily distinguishable by their lack of luminescence. ICE1 is a transcription factor that is able to recognise an element in the

promoter region of the *CBF3* termed the ICE1 Box (Fursova *et al.* 2009), and ICE2 binds to *CBF1* (Chinnusamy, Ohta et al. 2003; Fursova, Pogorelko et al. 2009). The *ice1* mutant has been shown to block induction of *CBF3*, however it has little effect on *CFB1*, whereas the *ice2* mutant affects *CBF1* expression (Chinnusamy *et al.* 2003, Fursova *et al.* 2009). A similar protein has been suggested named ICE1-like, which may potentially activate the ICE1 Box of CBF1 and CBF2 (Chinnusamy *et al.* 2007).

ICE itself is regulated by the gene high expression of osmotically responsive genes 1, (*HOS1)* (Ishitani *et al.* 1998). The *hos1* mutant was discovered via a mutant screen, and displayed increased induction of both *CBFs* and of downstream cold-induced genes under cold conditions. *HOS1* shows homology to a RING-finger protein; many of these proteins act as E3 ubiquitin ligases, and it was suggested that this is the role of HOS1 (Dong *et al.* 2006). The HOS1 protein was found in the cytoplasm at normal growth temperatures (Ishitani *et al.* 1998), and was proposed to negatively regulate ICE1 by targeting it for degradation by the 26S proteasome (Lee *et al.* 2001). *SIZ1* is the positive-regulator counterpart of HOS1, which sumoylates ICE1, stabilising it and allowing it to bind to the ICE1 box of CBF3, and activate gene expression (Miura *et al.* 2007).

1.8 Abscisic acid (ABA)

ABA is a phytohormone that is involved in a number of responses to abiotic stress; it has been suggested that it may mediate all environmental responses (Chen *et al.* 1983). An increase in ABA results in a number of physical changes to plant tissue, including the inhibition of growth and stomatal closure to reduce transpiration (Seo and Koshiba 2002).

Some groups have suggested that treatment of plants with ABA can lead to enhanced freezing tolerance (Lang *et al.* 1994), and a large number of genes have been found to be induced by both cold and ABA (Seki *et al.* 2002). However, it has also been noted that this rise in ABA on exposure to cold is transient, and within 48 hours it has returned to almost normal, whereas at this point the levels of freezing tolerance are still increasing (Lang *et al.* 1994). Both drought and freezing stress have been shown to lead to an increase in the cellular concentration of ABA (Mantyla *et al.* 1995).

The ABA deficient mutant, *aba-1,* is unable to produce ABA, and ABA-insensitive mutant (*abi1)*, which can manufacture but not respond to ABA, both display reduced freezing tolerance (Gilmour and Thomashow 1991), which suggests that ABA does have a role in the acclimation process. However, Thomashow (2010) subsequently argued that this reduced freezing tolerance may be due to the general poor health of the *aba-1* and *abi1* mutants; their reduced cold acclimation is more likely to be a result of the numerous other effects of being either ABA-deficient or unable to respond to it.

1.8.1 ABA-independent and ABA-dependent cold acclimation

It has been previously suggested that cold acclimation occurs via an ABA-dependent or an ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki 1994). ABA-dependent cold acclimation is said to require the activation of the ABA response element (ABRE), a *cis*-element found in the promoter of certain genes (Guiltinan *et al.* 1990). AREBS (ABA-response element binding factors) bind to the ABRE, activating the genes which have this element (Uno *et al.* 2000).

The ABA-independent pathway for cold acclimation was previously defined as being mediated by the CBFs. Certain *COR* gene promoters have been found to contain ABREs, including *COR15a* (Baker *et al.* 1994), Low-Temperature Induced 78 (*LTI78)* (Nordin *et al.* 1993) and Protein Kinase 2 (*KIN2)* (Kurkela and Franck 1990). It was thought that these could be activated by ABA without the input of the CBFs. However, it has since been discovered that ABA can activate the CRT and this may occur via *CBF1-3* (Knight *et al.* 2004). Studies have shown that whilst the CRT is activated by ABA, this does not occur at levels that alone induce cold acclimation (Knight *et al.* 2004). It is still open to debate as to how major a role ABA has in freezing tolerance; however, the role it plays in drought tolerance is well characterised (Seo and Koshiba 2002).

The induction and regulation of freezing tolerance is an extremely complex process. A great number of individual components have been proven to directly impact freezing tolerance in Arabidopsis and other species; however, there is a much lower level of knowledge concerning how each individual component links to another. In figure 1.1. both the known and postulated links between the main regulators of the freezing tolerance pathway have been highlighted.

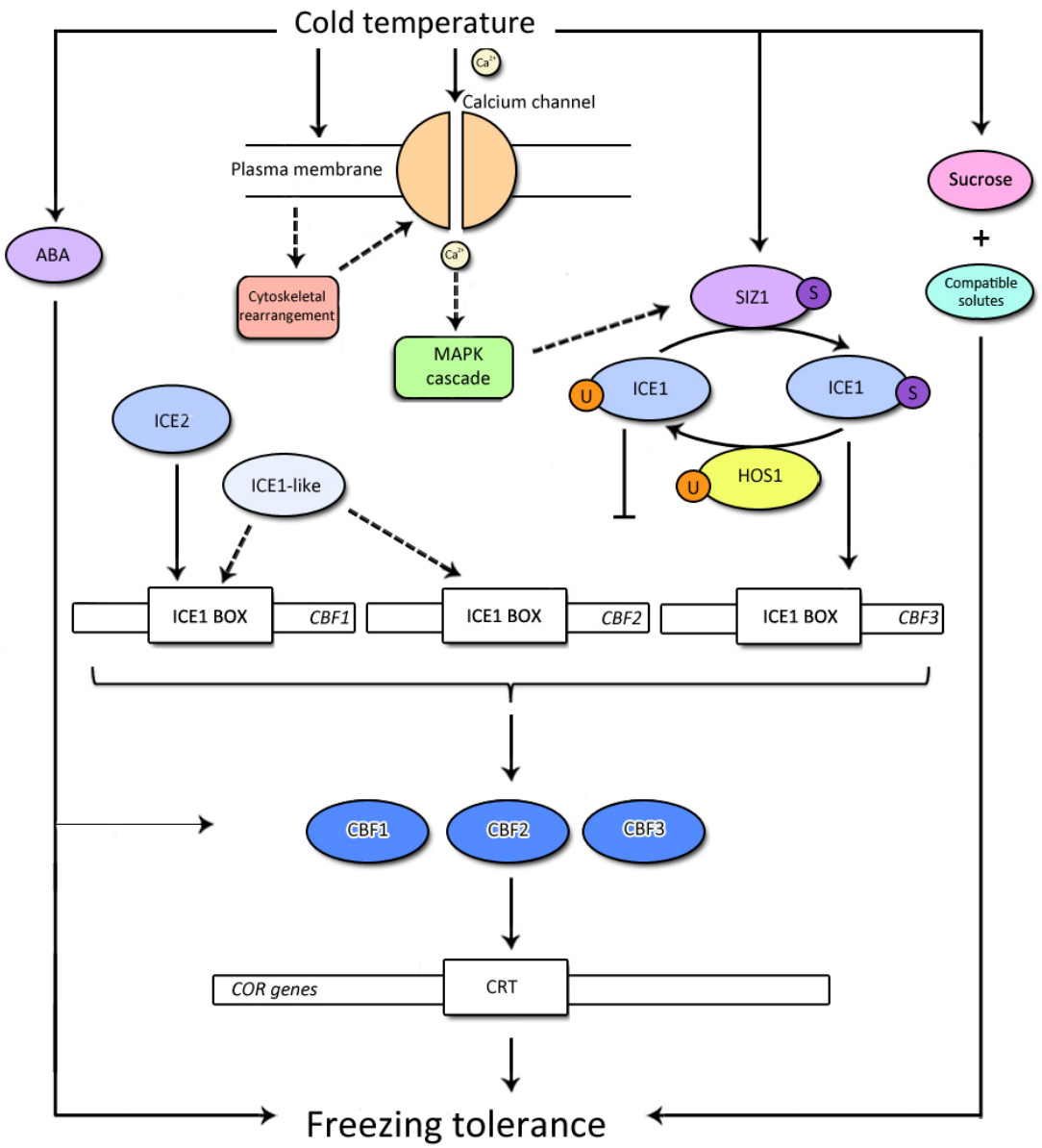


Figure 1.1. Theorised pathway of cold acclimation. Solid lines represent confirmed interactions; dotted lines represent theories that have not been biologically proven. Cold temperature is sensed by the membrane, which destabilises the cytoskeleton. This feeds back to the membrane and results in the opening of calcium channels. Calcium enters the cell and this activates a MAPK cascade. How the cascade affects other aspects of the pathway currently remains unclear, but it may link to SIZ1*. Sumoylation of* ICE1 *by* SIZ1 *allows* ICE1 *to activate the* CBF *genes (via the ICE1-BOX), which then brings about activation of COR genes, and with them, tolerance to freezing. When* ICE1 *is not required it is ubiquitinated by* HOS1 *and degraded. ABA has been shown to weakly induce expression of the CBFs, but also regulates freezing tolerance independently of the* CBFs*. However, it is important to note that alongside gene expression, there are other occurrences that bring about freezing tolerance, such as alterations in morphology, and the accumulation of compounds such as sucrose and compatible solutes.*

1.9. Cross-talk between the abiotic stress tolerance pathways

There are various ways in which the abiotic stress tolerance pathways overlap. The signalling components for each pathway are often shared between drought, cold and high salinity, as seen in figure 1.2. cold and drought stress essentially result in dehydration stress, so it is not unexpected that the two pathways may show cross-talk. Dehydrins, likewise, are present in response to drought (their role in freezing has been discussed previously) to protect cells from desiccation (Beck *et al.* 2007).

An increase in the level of cytosolic calcium is seen for drought, high salinity and cold (Knight 1999), similarly, the CDPKs previously mentioned have also been seen to be involved in the perception of other stresses (Urao *et al.* 1994). Reactive oxygen species (ROS) are molecules that are released due to stress and can cause damage to cells, however plants are able to use ROS as a form of signalling, and are capable of continually sensing the presence and levels of ROS (Miller *et al*. 2008).

Certain genes contain the ABRE and the CRT/DRE, an example of which, is the gene Responsive to Desiccation 29a (*RD29a,* also known as *LTI78*), proving that certain stress genes can be activated via different pathways, in the case of this gene, cold drought and high salinity (Yamaguchi-Shinozaki and Shinozaki 1994, Narusaka *et al.* 2003). The induction of one gene in response to a variety of stresses would indicate that plants mediate the damage caused by those stresses, to some extent, in the same way. In nature one stress is unlikely to occur alone, and as a result plants have to deal with the combination; the potential cross-talk between the different stress tolerance pathways may be a result of this.

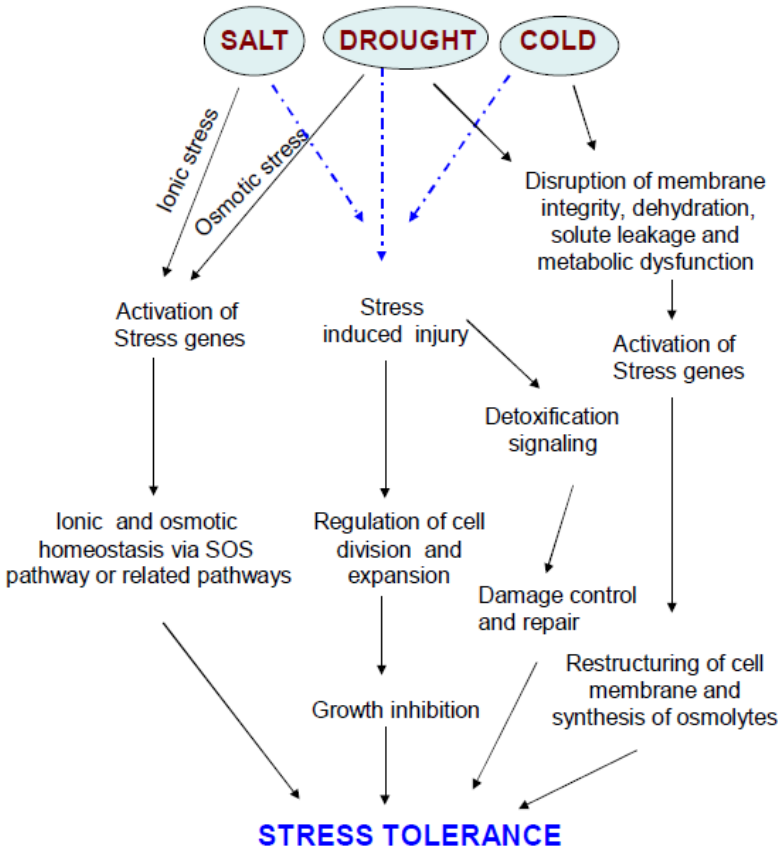


Figure 1.2. Cross-talk between abiotic stress tolerances. Diagram indicating the various modes of action in which plants respond to and counteract abiotic stress. Responses between the three main abiotic stresses are linked. Image from Mahajan et al. *2002.*

1.10. The *sensitive to freezing* mutants

The original 1-7 *sfr* mutants were isolated from a population that had been subjected to ethymethylsulfamate (EMS) chemical mutagenesis (James and Dooner 1990). The *sfr* mutants were distinguished by the way of a cold acclimation screen; when acclimation had taken place, the *sfr* mutants showed different levels of freezing tolerance when compared to healthy wild type Columbia 0 (Col-0) plants (Warren *et al.* 1996). Two further mutants (*sfr8* and *sfr9*) were identified at a later date, displaying the same reduced protection from freezing damage following acclimation (Thorlby *et al.* 1999). Classical mapping determined which chromosome each mutation was located on (McKown *et al.* 1996), and the mapping

intervals for each mutant were further refined at a later date (Thorlby *et al.* 1999). For each mutant only one allele is currently available; when *sfr5* was initially isolated, there were two mutant alleles; however seeds for *sfr5-2* are no longer available (Warren *et al.* 1996). Other than the noted sensitivity to freezing post-acclimation, there seem to be no pleiotropic effects of the mutations (McKown *et al.* 1996). Experimental data has shown that *sfr4* does not accumulate sucrose, glucose or anthocyanin to the extent that wild type Arabidopsis does in response to cold acclimation (McKown *et al.* 1996). A causal relationship has been demonstrated between the reduced sugar content of the mutant and its lack of freezing tolerance; supplementing the plant with sucrose results in the restoration of freezing tolerance (Uemura and Steponkus 2003). For other mutants, such as *sfr5,* there is no discernible reason as to why it is sensitive to freezing, suggesting that it may be governed by a novel pathway.

1.10.1. *sfr2*

sfr2 showed no pleiotropic effects and did not lack tolerance for any other abiotic stress (McKown *et al.* 1996). The sensitive to freezing phenotype that is seen is particularly strong for the whole plant assay, but much weaker for the electrolyte leakage assay (McKown *et al.* 1996). The mutation was mapped to chromosome III (McKown *et al.* 1996). An 11kb region of Col-0 DNA where the mutation was mapped to was transformed into *sfr2* plants; this was shown to restore freezing tolerance. Two genes were found in this region, and as a result these were sequenced, with a SNP being identified in one of the genes; a G to A transition that is most commonly seen with EMS mutagenesis, resulting in a missense mutation occurring at a very conserved amino acid within the gene At3g06510. The assumed loss-of-function was subsequently proven by the creation of other alleles (Thorlby *et al.* 2004).

The SFR2 protein was initially thought to be a constitutively expressed β-glucosidase (Thorlby *et al.* 2004), however this description was later altered to a galactolipid:galactolipid galactosyltransferase (Moellering *et al.* 2010). β-glucuronidase (GUS) staining localised SFR2 to predominantly green tissues, and further work has proven that it localises to the outer membrane of the chloroplast via the use of green fluorescent protein tagging and light microscopy, particularly in stomatal guard cells (Fourrier *et al.* 2008). The N-terminus has been proven to be responsible for targeting SFR2 to the outer chloroplast membrane. Immunoblotting was used to confirm that the C-terminus of the protein is located across the membrane. Whilst the gene responsible for the *sfr2* mutation has been discovered, the mechanism involved in confirming freezing tolerance is currently unknown. It has been suggested that it may modify the envelope of the chloroplast somehow to increase freezing tolerance, however, this is just speculation (Browse 2010).

1.10.2. *sfr3*

sfr3 shows the greatest freezing sensitivity in young leaves, specifically those that have not yet fully expanded (McKown *et al.* 1996). It has been shown that they are not compromised in the expression of certain *COR* genes (Knight *et al.* 1999). *sfr3*, as with several of the other *sfr* mutants identified in this screen, also shows reduced anthocyanin accumulation during acclimation (Thorlby *et al.* 1999). Classical mapping placed the mutation to the centromeric region of chromosome I (Thorlby *et al.* 1999). Due to this there were problems in mapping the mutation to a fine resolution, however this region contained few genes; those that were pseudogenes or transposable element genes were discarded (Amid *et al.* 2012). T-DNA insertion lines were analysed for the genes which remained. Nine genes were sequenced

using mutant *sfr3* DNA, and these were compared to the known annotated gene sequence. In one of the genes there was found to be a G to A mutation, which introduces a missense mutation into the coding region and creates a restriction site difference in gene *At1g36160* (Amid *et al.* 2012). This was the known gene *ACC1*, Acetyl CoA-carboxylase. This gene has been shown to be embryo lethal in a strong allele, however a weak allele (*glossyhead*) has been discovered that is not embryo lethal (Lü *et al.* 2011). This allele shows altered biosynthesis in the cuticular wax membrane. *sfr3* has been complemented with *ACC1* and this restored the freezing sensitivity entirely (Amid *et al.* 2012). Staining of plant leaf tissue revealed that *sfr3* plant tissue is damaged in response to freezing. It was also noted that wax crystals usually deposited on wild type stems when grown under cold conditions were not seen on *sfr3* inflorescences (Amid *et al.* 2012). This suggests that cuticular wax differences render Arabidopsis leaves unprotected under freezing conditions, resulting in the freezing injuries seen.

1.10.3. *sfr6*

Identified in the same freezing tolerance screen as *sfr2* and *sfr3*, *sfr6* appeared to be somewhat different from the other *sfr* mutants, with distinguishable yellow leaves (McKown *et al.* 1996). Unlike the other members of this screen it was shown that *COR* gene expression was reduced; this was proven both by RNA blot analysis and quantitative real time PCR (qRT-PCR). It was also proven that this reduction in *COR* gene expression (specifically protein kinase 1 [*KIN1*]) was linked to the phenotype. The reduced gene expression, like the phenotype, was also found to be recessive (Knight *et al.* 1999). The defect in *COR* gene expression was found to occur downstream of CBF transcription factor expression and appeared to be associated specifically with the expression of genes

regulated via the CRT motif (Boyce *et al.* 2003). Transcripts of genes with the CRT in their promoters failed to accumulate correctly.

The gene responsible for the *sfr6* mutation was identified by a combination of techniques (Knight *et al.* 2009) A mapping population crossed with Landsberg *erecta* (Ler) and coarse classical mapping mapped the mutation to a 44Mbp interval on chromosome IV, however, it was found to be close to the centromere which can cause problems due to low levels of recombination around centromeric regions. As a result, other methods were required to determine the identity of *sfr6.* Transfer DNA (T-DNA) insertion lines were obtained for genes between the two markers which *sfr6* was found to exist. Due to the unusual appearance of *sfr6* seedlings it was possible to visibly identify another allele of *sfr6* from the T-DNA-tagged populations (Knight *et al.* 2009). Two lines were found containing an insert in *sfr6* which included an insert in At1g04920 (Knight *et al.* 2009). The locus of this gene was sequenced from the *sfr6* mutant, and a G to A mutation was located in this gene, which introduced a stop codon, truncating the protein significantly (Knight *et al.* 2009).

SFR6 was identified as the subunit Mediator16 (MED16) of the Mediator complex, a transcriptional co-activator (Bäckström *et al.* 2007). In eukaryotic organisms, Mediator forms a bridge between a transcription factor and RNA polymerase II, bringing positive or negative regulation of gene expression, depending upon the stimulus (Conaway and Conaway 2011). The ideas of SFR6 being part of a transcriptional regulatory complex explains the extra defects that *sfr6* exhibits in addition to its cold-related phenotype; it displays deficiencies in gene expression for UV resistance and pathogen response, and also altered circadian rhythms (Knight *et al.* 2008, Wathugala *et al.* 2012).

1.11. Experimental aims

The aim of the work was to build upon the knowledge gleaned from the successful identification of *sfr2*, *sfr3* and *sfr6*; I wished to find the gene responsible for the reduced freezing tolerance in the mutants *sfr4*, *sfr5*, *sfr8* and *sfr9*. The advent of next generation sequencing technologies presented the opportunity to sequence entire genomes with relative ease. Compared to conventional mapping and crossing techniques, this vastly reduces the amount of time required to search for the SNPs which cause mutations in EMS mutants. This investigation specifically used Illumina next generation sequencing, and genome sequence data was compared to the known TAIR 10 reference genome. From this, SNPs were isolated within the known mapping regions for each mutant, and their potential impact on freezing tolerance was examined.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals other than those mentioned were sourced from BDH Merck Ltd. (Dorset, UK) or SIGMA-Aldrich (Dorset, UK).

2.2 Plant tissue

2.2.1. *sensitive to freezing* mutant seeds

All four *sfr* mutants used in this study were originally isolated by screening an ethyl methanesulfonate (EMS)-mutagenised population of Columbia 0 (Col-0) Arabidopsis (James and Dooner 1990) for plants unable to cold acclimate to freezing temperatures (McKown *et al.* 1996, Warren *et al.* 1996, Thorlby *et al.* 1999).

2.2.2. T-DNA insertional mutants

SALK lines (Salk Institute for Biological Studies, San Diego, USA) (Alonso *et al.* 2003) and GABI-Kat lines (Genomanalyseimbiologischen System Pflanze - Max-Planck Institut für Molekulare Pflanzenphysiologie, Potsdam, Germany) (Kleinboelting *et al.* 2012) contain an insert of transfer DNA (T-DNA) within a gene, and this insert is potentially capable of disabling that gene. The T-DNA insert in SALK lines contained the plant-selectable marker *NPTII (Neomycin phosphotransferase II)*, and was introduced via *Agrobacterium tumefaciens*. The *NPTII* marker introduced kanamycin resistance, and the T_2 progeny of the original T_1 transformants were selected for resistance to kanamycin (Azpiroz-Leehan and Feldmann 1997). GABI-Kat lines were created via a very similar method, but contain a *SULr* (*sulfadiazine resistance)* marker (Rosso *et al.* 2003). The Nottingham Arabidopsis Stock

Centre (Nottingham, UK) dispatched segregating T_3 lines for all SALK and GABI-Kat T-DNA mutant lines required for this investigation. The genotyping of these lines is described in section 2.9.2.1.

2.2.3. Seed sterilisation

The required number of seeds was placed into a 1.5ml microcentrifuge tube and 70% ethanol was added to sterilise. The seeds and ethanol were vortexed in a low-speed bench-top vortex (Labnet, Oakham, UK) for approximately five minutes to aid surface sterilisation. Seeds were then pipetted onto 90mm sterile filter paper circles (Whatman PLC., Maidstone, UK) inside a laminar flow cabinet (AstecMicroflow, Hampshire, UK) and the ethanol was allowed to evaporate off. Once dried, the seeds were dispensed onto agar plates containing growth media.

2.3. Growth media

2.3.1. Murashige and Skoog media

Standard full strength Murashige and Skoog media (Murashige and Skoog 1962) containing vitamins (Duchefa Biochemie, Ipswich, UK) was used for initial plant growth. One litre of milli-Q water was combined with 4.4g of powdered media to give a 1x MS solution. The pH of the solution was brought to 5.8 by addition of 0.1M KOH. Plant tissue culture grade agar (SIGMA-Aldrich, Dorset, UK) was added to a final concentration of 0.8%, and the mixture was then autoclaved (121°C, 1.03 x 10^5 P_a for 20 minutes). When cooled to approximately 50°C the media was poured into Petri dishes in the laminar flow hood. Lids were replaced as

the media was allowed to solidify. Two sizes of dish were used: 90mm (Scientific Laboratory Supplied Ltd, Hessle, UK) and 55mm (Fisher Scientific UK Ltd, Loughborough, UK).

2.3.2. Mannitol media

Media containing mannitol was made to the same specifications as in section 2.3.1, however varying amounts of mannitol (BDH Merck Ltd., Dorset, UK) were added to the MS solution before the pH was adjusted and the solution was autoclaved. Mannitol media was made up to 200mM 300mM, 400mM and 500mM mannitol concentrations for seedling emergence (section 2.6.1.).

2.4. Growth conditions

2.4.1. Standard growth conditions

Seeds were sown onto Petri dishes containing MS media, and were allowed to stratify in a 5°C cold room for a minimum of four days. After stratification the lids were taped down with Micropore tape (3M United Kingdom PLC., Brackwell, UK) and the seeds were transferred to a Percival growth chamber (Percival Scientific Inc., Perry, USA), set to 20°C (+ or - 1°C) light levels at 150µE m^{-2} s^{-1} for 16 hours, followed by eight hours of darkness. Here seedlings were left to grow for seven days, before being transferred to peat plugs (LBS Horticulture Ltd., Lancashire, UK) of either 44mm or 41mm in size; these were placed in opaque trays to aid watering. Plants required for seed were transferred to a long day growth room (20°C [+ or - 2°C], 16 hours light, 8 hours darkness, 150 – 200µE m^{-2} s^{-1}) to promote flowering. When plants began to flower the Arasystem (BETATECH bvba, Ghent, Belgium) was used to prevent cross-contamination of seeds between different lines. The plants were watered until no plant tissue remained green and tissue was allowed to desiccate prior to seed

collection. Once collected seeds were dried overnight at 37°C, and then stored at 5°C to preserve viability and germination rate. If plants were required for freezing assays they were grown under short day conditions, 20°C (+ or - 2°C), 12 hours light, 12 hours darkness and between 150 and 200 µE m^{-2} s^{-1} to encourage rosette growth and suppress flowering.

2.5. Freezing treatment conditions

2.5.1. Standard adult plant freezing assay

Seeds were sown onto Petri dishes containing MS media, and were grown under standard growth 'short day' growth conditions for five weeks. At this point they were relocated to a SANYO MLR-351 environmental test chamber (Sanyo, E&E Europe, BV, Biomedical division, UK) set to 5°C, 150 µE m^{-2} s^{-1}, 10 hours light, 14 hours darkness, mimicking the conditions plants would experience during cold acclimation. After 10-14 days, half of the available the plants were transferred to a SANYO freezing chamber (MIR-254) (Sanyo, E&E Europe, BV, Biomedical division, UK), set at -7.5°C for 24 hours; the other half remained at 5°C. During this time plants were in darkness. After freezing, plants were transferred to a SANYO environmental test chamber set to 5°C for approximately 30 minutes before being returned to the 20°C 'short day' growth room where they were monitored for re-growth. Col-0 plants that were capable of cold acclimation were able to survive this treatment, whilst known *sfr* mutants showed poor survival rates. The re-growth of these plants was photographically documented.

2.5.2. T-DNA insertional mutant adult plant freezing assay

Post-genotyping (2.9.2.1), populations of homozygous T-DNA lines were tested for freezing sensitivity under the conditions listed above. Each T-DNA line was tested alongside the original EMS *sfr* mutant to which the T-DNA line corresponded, and with Col-0 plants as a baseline for freezing tolerance. If the T-DNA line responded to freezing conditions in a similar manner to the *sfr* mutant (i.e. little or no recovery after freezing) further work was conducted on these lines as the gene affected by the T-DNA insertion could be responsible for the freezing sensitivity seen in the *sfr* mutant.

2.5.3. Petri dish freezing assay

Col-0 and *sfr6* seeds were sown onto Petri dishes containing MS media, and were grown under standard growth conditions for seven days. Seedlings were then transferred to a SANYO environmental test chamber set to 5°C, 150 µE m^{-2} s^{-1}, 10 hours light, 14 hours darkness, for four days. At this point the seedlings were transferred to -7.5°C for 24 hours. During the freezing stage Petri dish lids were removed to aid airflow and ice nucleation. Post-freezing, seedlings were then returned to the 5°C SANYO chamber for 30 minutes, and then to the 20°C Percival chamber and were observed for re-growth after two days.

2.5.4. Cold treatment of seedlings for assessment of cold-inducible gene expression

Col-0, *sfr4*, *sfr5* and *sfr8* seeds were sown onto Petri dishes containing MS media; four petri dishes were sown for each seed line. Seedlings were grown up under standard growth conditions for seven days. At this point, two plates of each line were transferred to a SANYO chamber set at 5°C, and two to a SANYO chamber set at 20°C. The plants were subjected to light levels of 150 µE m^{-2} s^{-1} for the duration of their stay in the chamber. One plate of each

seed line was removed from each of the chambers after two hours, and approximately 30 seedlings were transferred to a 1.5ml microcentrifuge tube and were frozen in liquid nitrogen. The transfer from agar to liquid nitrogen was undertaken in less than one minute to preserve the RNA. The remaining plates were removed from the chambers after six hours, and were extracted from the agar and frozen in the same manner. The frozen plant tissue was then subjected to RNA extraction (section 2.8.3) and cDNA synthesis (section 2.8.4) for use in quantitative real time PCR, with the aim of measuring changes in *CBFs*, *KIN2* and *GOLS3* gene expression in the *sfr* mutants and Col-0.

2.6. Other stress growth conditions

2.6.1. Osmotic stress tolerance: seedling emergence

Col-0, *sfr4*, *sfr5*, and *sfr8* seeds were sown onto Petri dishes containing MS agar supplemented with differing concentrations of mannitol: 0mM, 200mM, 300mM, 400mM and 500mM. The details of how this media was made are available in section 2.3.2. The seedlings were grown for seven days under standard growth conditions. For each mannitol concentration, there were five repeats for each seed line. At seven days of age, radicle emergence was monitored using a light microscope with a 40x lens, and percentage emergence was recorded for each plate recorded.

2.6.2. Osmotic stress tolerance: whole seedlings

Col-0, *sfr4, sfr5* and *sfr8* seeds were sown onto Petri dishes containing MS agar and were grown for seven days under standard growth conditions (section 2.4.1). At seven days of age the seedlings were extracted from the MS medium and transferred to a 24-well flat bottom suspension plate (Sarstedt, Newton, USA). Seedlings of each line were floated on 1.5 ml of

0mM, 330mM, 440mM and 550mM mannitol. Seedlings were transferred to a Percival growth chamber set to 20°C (+ or - 1°C) light levels at 150µE m^{-2} s^{-1} and remained there for 72 hours. After this time the seedlings were photographed, and the level of cholorsis for each line was judged.

2.6.3. Measurement of sucrose and light-regulated gene expression

Col-0, *sfr4* and *sfr8* seedlings were sown onto Petri dishes containing MS agar and were grown under standard growth conditions for seven days. At this point they were extracted from the agar and were floated on two different solutions in both light and dark. 0.088M sucrose was the chosen test condition, with an iso-osmolar concentration of the non-metabolisable sugar mannitol (0.096M) to control for any osmotic stress caused by the increased level of sucrose. Half of the seedlings were kept in the light throughout the experiment, and the remainder were transferred to the dark for six hours, three hours after subjective dawn. Quatitative real time PCR measured the dark-induction and sucrose-repression of *DIN6*.

2.7. Observational studies

2.7.1. Flowering time assay

Col-0 and *sfr8* seedlings were sown on MS agar plates and grown under standard long day conditions in the Percival growth chamber. At seven days old the seedlings were transferred to peat plug and grown under long day conditions. Individual plants were monitored every day for signs of flowering; when a flower bolt was 1cm long the number of leaves on that

plant was noted, as was the day (Lee *et al.* 1993, Knight *et al.* 2008). The results of each individual were tabulated and statistically analysed using the Student's *t*-test.

2.8. Nucleic acid extraction

2.8.1. Extraction of genomic DNA for next generation sequencing

The initial sequencing of the *sfr* mutant genomes was conducted using genomic DNA extracted via the Qiagen Plant Mini Kit (Qiagen Ltd., Crawley, UK). This was conducted before the commencement of this project by Dr. Heather Knight. For the re-sequencing of *sfr4* a modified version of the CTAB (Cetyltrimethylammonium bromide) DNA extraction protocol (Richards *et al.* 2001) was used.

For the CTAB extraction method approximately 100mg of tissue was frozen in liquid nitrogen and ground in a pestle and mortar filled with liquid nitrogen. 800µl of CTAB extraction buffer (2% CTAB, 1.42M NaCl, 20mM EDTA, 100mM TrisHCl, 2% PVP 40, 5mM Ascorbic acid, 4mM DIECA [diethyldithiocarbamic acid]) was heated to 60°C and added to the plant tissue. The tissue was then transferred to a 1.5ml microcentrifuge tube and 3µl of β-mercaptoethanol was added. The tube was inverted and incubated at 60°C for 30 minutes. 500µl of cholorphorm:iso-amyl-alcohol (24:1) was added and mixed by inversion, before being centrifuged at 15,600*g* for five minutes. The supernatant was removed and the pellet allowed to air dry in a heated block set at 37°C for approximately 30 minutes. DNA was re-suspended in 100µl of TE buffer, and RNase A was added to a concentration of 10µg/µl. The sample was incubated at 30°C for 30 minutes. 500µl of cold isopropanol, was added, mixed by inversion and precipitated at -20°C for 30 minutes.

The sample was centrifuged at 15,600*g* for ten minutes and the supernatant was discarded. The DNA pellet was washed in 800µl of 70% ethanol for 20 minutes and spun at 15,600*g* for five minutes. The supernatant was removed and the sample was air dried at 37°C for 30 minutes. 100µl of TE buffer was used to re-suspend DNA and the sample was left overnight a 4°C. The sample was then sent to The Genome Analysis Centre (Norwich, UK) for further processing, including sonication and preparation for next generation sequencing.

2.8.2. Extraction of genomic DNA for PCR

This method is based on that detailed in Edwards *et al.* 1991, however, several alterations have been made. Approximately six seven day old seedlings (or one rosette leaf of an adult plant) were transferred to a 1.5ml microcentrifuge tube and were immediately frozen in liquid nitrogen. Leaf tissue was manually macerated using a micropestle. After approximately five seconds 400µl of Edward's extraction buffer (250mM NaCl, 0.5% SDS, 25mM EDTA pH 8.0, 20mM Tris-HCl pH 7.5) was added. Once complete for all samples, they were spun for approximately one minute at 16,300*g* in a Genfuge 24D Microcentrifuge (Progen Scientific, London, UK). 300µl of supernatant was removed from the sample and transferred to a clean 1.5ml microcentrifuge tube, to which 300µl of isopropanol was added. The samples were mixed by inversion and a two-minute bench top incubation was carried out to aid the precipitation of DNA. The samples were spun for ten minutes at 16,300*g* and the supernatant was aspirated. All samples were spun under vacuum for approximately five minutes in an Eppendorf Concentrator 5301 (Eppendorf UK Limited, Stevenage, UK) to dry. 50µl of TE buffer (1mM EDTA, 10Mm, Tris-HCl pH8) was added to each sample, and all were

left overnight at 4°C to allow the DNA to re-dissolve. Samples were then transferred to -20°C for long-term storage before use.

2.8.3. Extraction of RNA

The Qiagen RNeasy Mini RNA extraction kit (Qiagen Ltd., Crawley, UK) was used to extract RNA from plant tissue. RNase-free filter tips and tubes were used throughout the procedure. Tissue samples were transferred directly from -80°C to a Dewar of liquid nitrogen. Each sample was individually removed from the Dewar and manually macerated using a micropestle (Anachem Ltd., Luton, UK). 450µl of RLT lysis buffer (with β-mercaptoethanol added) was added to each sample, and a motorised micropestle was used to homogenise the tissue. The sample was vortexed and heated to 56°C in a heat block (Labnet, Oakham, UK) for between one and three minutes, and were then transferred to ice for a minimum of five minutes. The lysate was transferred to a QIASHREDDER column to aid the shredding the plant tissue and allow separation of cell lysate from cell wall materials, and was then centrifuged for two minutes at 16,300*g*.

The purified lysate from the collection tube was transferred to a fresh collection tube, and combined with 225µl of 100% ethanol, before being transferred to an RNeasy spin column and associated collection tube. The new column was centrifuged for 30 seconds at 11,400*g*. Discarding the flow through, 350µl of RW1 wash buffer was added, and the column was centrifuged once again for 30 seconds at 11,400*g*, discarding the flow-through.

Per sample, 10µl DNase I stock solution and 70µl of Buffer RD were added to the column membrane and were incubated for 20 minutes, to remove genomic DNA which could have

contaminated the sample. 500µl of was buffer RPE was added to wash, and all columns were centrifuged for 30 seconds at 11,400*g*. This step was then repeated. The flow-through was discarded, and the column was spun at 11,400*g* for two minutes. RNA was eluted in 30µl of RNase-free water, centrifuged for one minute at 11,400*g*, and quality and yield of RNA were measured using a NanoDrop 1000 spectrophotometer (Nanodrop Products, Delaware, USA).

2.8.4. cDNA synthesis for qRT-PCR

The Applied Biosystems High Capacity cDNA synthesis kit (Applied Biosystems, Foster City, USA) was used to synthesise cDNA suitable for qRT-PCR. Reagents (2µl 10x RT buffer, 0.8µl 11mM dNTPs, 2µl 10x RT Random Primers, RNA) were thawed on ice. The amount of RNA was standardised between reactions to ensure 2µg was present in a 10µl volume. Each set of reactions was set up with a 'No Template Control', which contained water instead of RNA, and a 'No Reverse Transcriptase' control to assess genomic DNA contamination, (no cDNA is produced in this reaction therefore only contaminating genomic DNA is available as template for subsequent PCR amplification).

Samples were then transferred to a Thermal Cycler: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for five seconds, with a final hold step at 4°C. Samples are then stored at -20°C for use in qRT-PCR, and before use were diluted 1:50.

2.9. DNA

2.9.1. Primer design and synthesis

Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) was used to design primers for all PCR reactions including quantitative measurement of transcript levels, excluding those used for SALK and GABI-Kat genotyping. These were designed by T-DNA Express (http://signal.salk.edu/tdnaprimers.2.html). All oligonucleotide primers for PCR and qRT-PCR were ordered from Invitrogen (Life Technologies Ltd, Paisley, UK); all primer sequences are listed in the appendices.

2.9.2. Polymerase chain reaction

For all PCR applications, the DNA polymerase BioTaq RED Polymerase (Bioline, London, UK) was used; this product is a standard Taq polymerase without 3' to 5' exonuclease proofreading capability. Genomic DNA extracted using the above Edward's method (2.8.2) was usually used as the template for PCR, and the standard reaction mixture for each sample included, 1µl of each the forward and reverse primers (50µM), 1µl dNTPs (10mM), 1.5µl of $MgCl_2$ (50mM), 1µl of BioTaq RED polymerase, 5µl of Bioline 10x buffer (Bioline, London, UK), 1µl of genomic DNA, and the reaction was made up to 50µl with water. For genotyping, reactions were often reduced to 20µl as extraction of the sample from a gel was not required.

The standard PCR cycle consisted of a first stage: 95°C for 5 minutes, 55°C for 5 minutes followed by 72°C for 5 minutes, which was repeated once, and a second stage consisting of: 95°C for 1 minute of denaturation, 55°C for 1 minute of annealing, and 72°C for 2 minutes of extension of the transcript. This second stage was repeated between 30 and 35 times

depending on the template being amplified. There was a final stage of 72°C for 10 minutes, before the sample was held indefinitely at 4°C until further use.

2.9.2.1. Genotyping PCR

In SALK and GABI-Kat insertional mutant lines, a stretch of T-DNA has been inserted into a gene, and in doing so has disrupted the proper functioning of the gene, potentially knocking it out. T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress/) was used to identify available T-DNA insertion lines for the genes in question. These lines were grown up, and pre-designed primers from T-DNA Express were obtained for genotyping of individual plants. Genotyping PCR was conducted, with the expected results shown in figure 2.1.

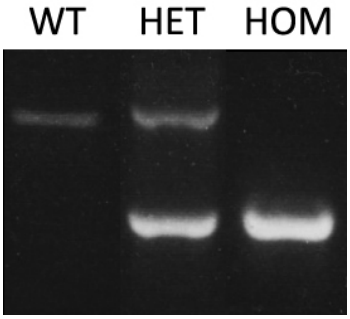


Figure 2.1. A representative image of genotyping PCR of T-DNA insertional lines. Wild type (WT), heterozygous (HET), and homozygous (HOM) banding patterns are indicated.

PCR was carried out to verify the genotype of each plant, with the possible outcomes being wild type, heterozygous for the T-DNA insertion, or homozygous for the T-DNA insertion. The primers designed to verify that the genomic sequence was uninterrupted at the locus are referred to as the 'genomic' primers; when used in a PCR reaction these would produce a product of the expected size for a wild type or heterozygous individual. A 'left border' primer which anneals to the T-DNA insert was used with the 'right' genomic primer; if this combination gives a PCR band, it proves that an insertion of the T-DNA has taken place. If

the T-DNA reaction band is the only band present, the plant is a homozygote for the insertion, however if both a genomic band and a T-DNA band are present the plant is a heterozygote. Col-0 genomic DNA was used as a positive control for the genomic reaction and a negative control for the T-DNA insert.

2.9.3. Gel electrophoresis

PCR samples were run on ethidium bromide agarose gels, ranging from 0.8 to 1.2%, to verify that products of the correct size had been produced. The required amount of molecular grade agarose (Bioline, London, UK) was added to a flask, and was made up with the correct volume with 0.5xTBE buffer to give a 1% w/v gel (45mM Tris-borate, 1mM EDTA). The flask was heated until the agarose had completely dissolved. Ethidium bromide was added to the final concentration of 5µg/ml, and the gel was poured into a gel casting tray and allowed to set. 5µl of Hyperladder I DNA ladder (Bioline, London, UK) was run in one lane of every gel. The samples were run at 35mA for one hour, and were visualised using a UV gel documentation system (Uvitech Ltd., Cambridge, UK).

2.9.4. Gel extraction

The agarose gel was placed on a UV transluminator and a clean scalpel was used to excise each band, Each sample was transferred to a 1.5ml microcentrifuge tube and was weighed. The QIAquick gel extraction kit (Qiagen Ltd, Crawley, UK) was used to extract bands from ethidium bromide gels to be sent for direct DNA sequencing. Three volumes of gel solubilisation buffer QG (containing guanidine thiocyanate) were added per volume of gel (100mg ≈ 100µl). The samples were incubated at 50°C for ten minutes, and one gel volume of isopropanol was added. Samples were transferred to QIAquick columns and collection

tubes, and centrifuged at 16,300*g* for one minute. 500µl of Buffer QG was added, and the spin was repeated. 750µl of Buffer PE was added to wash and prevent DNA from disassociating from the spin column, and samples were allowed to stand for between two and five minutes. The samples were centrifuged for one minute, the flow-through was discarded, and samples were centrifuged again to remove residual ethanol. DNA was then eluted in 30µl of buffer EB (10mM Tris.Cl pH8), allowing one minute standing before centrifuging. Concentrations of samples and contamination levels were then checked on a Nanodrop 1000 Spectrophotometer.

2.9.5. Direct DNA sequencing

All direct DNA sequencing was carried out using the Applied Biosystems 3730 sequencer, at the Durham DNA Sequencing and Fragment Analysis Facility (http://www.dur.ac.uk/biosciences/services/dna/).

2.10. Relative quantification of transcripts by quantitative real time PCR

Quantitative real time PCR was used to analyse the expression of of genes of interest. The process involved the quantification of PCR product that had been amplified with primers specific to that gene, using cDNA as the template for PCR. This gave a relative quantification of the cDNA corresponding to the gene of interest, and therefore a relative quantification of the amount of transcript (mRNA) present in the original sample. Expression was normalised to expression of *PEX4*, a constitutively expressed gene, to control for any differences in the amount of cDNA between samples (Wathugala *et al.* 2012). cDNA used in this assay was synthesised using reverse transcriptase (2.8.4).

Primers were designed to anneal to a ~100bp region of interest, and were used in the qRT-PCR assay. Where possible, they were designed to span an intron. An Applied Biosystems 7300 Real-Time PCR System was used for all assays. A 96-well plate (Starlab UK, Milton Keynes, UK) was used for the reaction, with each sample having three technical replicates, to assure that differences seen in gene expression were not as a result of pipetting inaccuracy. The number of biological replicates that were conducted for each experiment is indicated in the results; the optimum number of biological replicates for each experiment would be three, however due to time constraints it was not possible to conduct this number of separate experiments. This does have an effect on the statistical significance of these results, and for those experiments with only one biological replicate they can be considered no more than preliminary work. Those with two biological replicates that display the same expression pattern give results in which it is possible to have much more confidence, however, a third replicate would be the ideal; this is something that is suggested as further work.

Each well contained 7.5µl of SYBR Green 2X qPCRMastermix (PrimerDesign, Southampton, UK) 1µl of each primer (5µM), 1.5µl of water and 5µl of a 1:50 dilution of the initial cDNA reaction. The fluorescent dye SYBR green binds to newly synthesised double-stranded DNA and thus enables quantification of PCR products amplified during the assay. One set of cycling conditions were used for all qRT-PCR cycles conducted during this investigation. An initial 10 minute step at 95°C is followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by 95°C for 15 seconds, 60°C for one minute, and 95°C for 15 seconds. The final step, a dissociation step, allows the user to determine whether the primers are

amplifying the intended cDNA target or contaminant gDNA and indicate whether primer dimers have formed.

Results were analysed using the Applied Biosystems software associated with the machine (Version 1.4). The method of analysis used on qRT-PCR data was the $2^{-\Delta\Delta C}T$ (delta delta C_T) method (Livak and Schmittgen 2001). The software outputs a C_T (cycle threshold) value, which represents the number of cycles taken for the amount of fluorescent product to exceed an arbitrary threshold. Calculation of delta delta C_T values for each sample gives a relative quantification of gene expression after normalisation to the endogenous control gene *PEX4*, referred to as the 'RQ' value. Statistics were performed in accordance with the Applied Biosystems user bulletin 'Relative Quantification Algorithms in Applied Biosystems Real-Time PCR Systems Software'. In order to employ this method in which standardisation against a constitutively active endogenous control gene is used it must be assumed that the amplification efficiency of the control gene and the target gene are equal. This was verified via primer testing.

Before use, the amplification efficiency of new qRT-PCR primers must be estimated. C_T values from the qRT-PCR reaction were plotted against a serial dilution of cDNA; if primers are functioning efficiently, a doubling of cDNA concentration should result in a shift of one C_T unit. All primers used previous to this investigation have been verified to be successful; those designed in the course of this investigation were also verified, and the results of these verifications are included in the appendices. The results of qRT-PCR were displayed graphically, and error bars on histograms indicate RQ min and RQ max and constitute the acceptable error for a 95% confidence limit according to the Student *t*-test between these samples.

2.10.1. Testing cold-inducibility of candidate genes

qRT-PCR was used to see if the gene in question was inducible in response to cold. A candidate that did not appear to show cold-inducibility was not ruled out from further investigation, however a cold-inducible candidate may have suggested it was likely to be linked to the freezing sensitive phenotype.

Four wild type cDNA samples, (two hours at 20°C, six hours at 20°C, two hours at 5°C, six hours at 5°C) were tested with each set of real-time primers, and the level to which the transcript was detected at ambient and cold temperatures was compared.

2.10.2 Gene transcripts of T-DNA mutant lines

When a homozygous population of individuals was either received directly from the stock centre or isolated after genotyping (section 2.9.2.1) it was verified that the T-DNA insert was causing reduced levels of full-length transcript. This reduced transcript level would occur either due to the insertion occurring in the promoter region, hence reducing levels of normal transcript, or the insertion occurring in the coding region, interrupting and hence truncating the transcript. Insertions occurring in introns could also alter the splicing, affecting transcript levels.

Primers were designed for either side of the proposed site of T-DNA insertion, as to show that in a wild type sample the gene is functioning correctly, however in the insert line, the region of DNA would not be transcribed, as there would be a large insert in the middle.

2.11. Next generation sequencing and bioinformatics

2.11.1. Illumina next generation sequencing

Illumina next generation sequencing (NGS) involves the use of reversibly terminable fluorescent dyes, and produces a large number of short reads (Mardis 2008). It is idea for use in species where a reference genome has already been produced, and in this instance was used in the search for the single nucleotide polymorphisms (SNPs) responsible for each of the *sfr* mutations. This was carried out by The Genome Analysis Centre (Norwich, UK).

2.11.2. Analysis of next generation sequencing data via the Galaxy method

Galaxy (https://main.g2.bx.psu.edu/) is a free, open-source platform that allows the user to upload raw NGS data with the intention of mapping these data against a reference genome; The interface allows a variety of programs to conduct the mapping, and in this instance the mapping tool used was Bowtie for Illumina (https://main.g2.bx.psu.edu/). These data were mapped, using Bowtie's default settings, to the latest Arabidopsis reference genome, TAIR 10 (Lamesch *et al.* 2012).. This file-type that is outputted at the end of the process can be read on the Integrative Genomics Viewer (IGV) software (http://www.broadinstitute.org/software/igv/). This is a predominantly automated method of mapping, but does contain a level of customisability for the more advanced user. This method was used to create the .bam output files used in this investigation, and this was carried out by Professor Marc Knight.

2.11.3. Integrative Genomics Viewer (IGV)

The IGV software allows the resultant data from Illumina sequencing to be compiled and arranged into a format that allows the entire genome to be viewed and compared with the

most recent TAIR 10 Arabidopsis genome sequence (figure 2.2). The number of 'reads' conducted for each portion of the genome can also be seen, and it is evident when coverage is poor in areas of the genome, as the number available for a certain stretch of DNA is lacking. If a mutation is found in a high enough percentage of reads, the software will draw the user's attention to it, allowing them to pick out mutations that have a highest confidence of being 'true' and found in the DNA. This threshold can be manually set, and for this particular study, was set at 0.7, as the mutations being searched for are homozygous, and hence should appear in 100% of reads, however, this would not account for possible sequencing errors, and setting the threshold at 1.0 could lead to mutations that are legitimate being ignored.

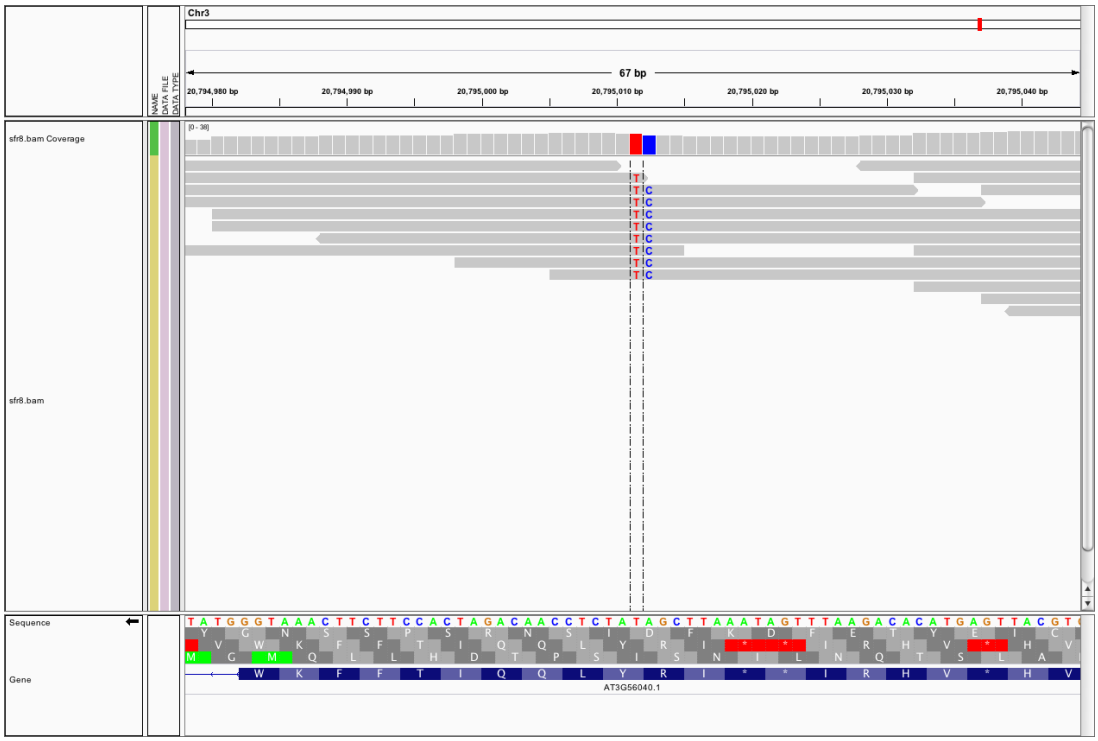


Figure 2.2. Example of two mutations viewed in the Integrative Genomics Viewer software. Bases that agree with the reference genome are coloured grey, however when a difference arises from the reference genome, the base is flagged up with a colour corresponding to which base it is.

As the mapping interval for each of the *sfr* mutants has been calculated (Thorlby *et al.* 1999), the software allows the user to manually scan through this specific region of the genome, noting down the position of potential causes of the mutation in each of the mutants. These mutations will be given an order of importance according to the following pre-determined set of criteria. It would be ideal to pursue all of the SNPs found by the software, even if they were deemed unlikely, however due to time constraints the number had to be reduced to the most likely candidates.

2.11.4. Selection criteria for mutations

2.11.4.1. Where the mutation is found

Mapping intervals for each of the *sfr* mutants had previously been determined, (Thorlby *et al.* 1999), and were subsequently updated in personal communications from Glen Thorlby. Mutations found outside these updated intervals were immediately discarded. The mapping intervals for each of the *sfr* mutants had been determined with a reasonable degree of accuracy, and it is highly likely that the mutations causing the sensitive to freezing phenotypes will be found within them.

Viewing the data on a finer scale, a mutation present in the coding region of a gene was treated with a much higher preference than one that is found elsewhere, as it is more likely to have a greater effect on the phenotype. It is possible that a mutation could occur in the promoter region, or a downstream UTR that controls transcript stability, and this could have a substantial effect on the gene, and while there could be the possibility of microRNAs – which are small, non-coding transcripts of RNA that bind to mRNA – having an impact on freezing tolerance, it is more likely that the cause of this mutation is a base change within a

gene. In the case of no mutations being found in genes within the interval, promoter regions would be the next step, as it can be argued that disrupting the promoter sequence of a gene will result in it not being activated.

2.11.4.2. Number of reads in which the mutation is present

If a candidate mutation is present in every read available for that region of genomic DNA, it is more likely that this mutation will be found in the DNA of the mutant plant, rather than being an error that occurred in the sequencing process. If a mutation is found in a region of the genome for which the data have only provided one read that has been repeatedly sequence there is a higher chance of this being a sequencing error, compared to reads that start and finish in different places. As a result, if, for one potential mutation, all of the reads are exactly the same length and have the same starting position they are counted as the 'same' read, and this greatly reduces the reliability of this SNP. While it should not be discounted, such mutations were put aside in favour of mutations which fulfilled more of the criteria, as this was only a one-year project.

2.11.4.3. Read direction

The direction of read is important; if a mutation is found in reads facing in only one direction, it is much more likely to be a sequencing error. If a mutation is found in various reads facing in both directions this increases the confidence that it is legitimate, however it also has to be take into account that it could just be co-incidence that reads facing in one direction do not show the mutation, and is not a strong enough criterion to reject a mutation purely on this basis.

2.11.4.4. The type of mutation

The original *sfr* mutants were generated by EMS, a chemical mutagen that introduces SNPs. It has been statistically proven that the vast majority of EMS mutations are from G or C to A or T, however the *sfr* mutation could be the result of a reversion event caused by the EMS. There is also the possibility that the mutation could have occurred independently of the EMS mutagenesis. As a result all base changes will be considered.

2.11.4.5. The result of the mutation

The SNP found in the IGV viewer and its position is located within the known coding DNA sequence of the gene. The effect that the mutation has on the protein sequence is shown by altering the amino acid in question and running it through the ExPASy online web application (http://web.expasy.org/translate/). The program gives the predicted translation for the sequence of the mutant DNA. This was compared to the translation that the un-mutated Col-0 translation.

2.11.4.6. The presence of the mutation in the DNA of the mutant plant

By this point the number of potential candidates for the mutation will be greatly reduced, and at this point PCR can be conducted to verify the existence of mutations within the genomic DNA of the mutant plants. Genomic DNA was extracted from the plant, and primers were designed to allow the amplification and sequencing of the portion of DNA which contained the putative mutation. Direct DNA sequencing took place, and using NCBI BLAST the resultant sequence was compared to the known DNA, and if there was a difference between the nucleotides of the wild type sequence and the mutant, this sample

was processed further. If the mutation was not present in the DNA, the potential mutant was discarded, as it was likely to have been an error during the Illumina sequencing.

One *sfr8* candidate mutation was ruled out at this stage, as it was not found to be present in the DNA of the mutant plant. This highlights the importance of not relying on the software, as sequencing errors do occur and can appear to be very convincing.

2.11.5. Analysis of next generation sequencing data via the command line method

Some data – specifically *sfr5* – did not map well using the Galaxy tool, and as a result was analysed in a different manner. Laura Gardiner of the University of Liverpool used a command line approach rather than an automated system to 'call' SNPs. This involved a number of Linux command line-operated bioinformatics programs, BWA (Burrows-Wheeler Aligner) (Li and Durbin 2009), SAMtools (Sequence Alignment/Map) (Li *et al.* 2009), GATK (Genome Analysis Toolkit) (McKenna *et al.* 2010, DePristo *et al.* 2011) and the 'awk' command. These allowed fine-tuning of the criteria each SNP had to abide by to be considered legitimate. The method outputs a list of SNPs, which are possible homozygotes and heterozygotes. For the purpose of this experiment, only homozygous SNPs are of interest.

2.11.6. The Arabidopsis Information Resource (TAIR)

Various tools provided by TAIR (http://www.arabidopsis.org/) were used; the gene database was used to verify if any of the candidate genes had been the subject of previous study. The SeqViewer was used to determine what area of the genes certain mutations existed within (promoter, etc). GBrowse was used to determine whether SNPs found via the command line method were within genes, as these data were supplied as genetic co-ordinates.

2.11.7. NCBI Basic Local Alignment Search Tool (BLAST)

BLAST, specifically nucleotide blast was used to compare the known sequence of a candidate gene with that sequenced from PCR product of the mutant DNA, to see if the reference allele of the Col-0 gene was different to that of the mutant gene.

3. RESULTS

3.1. Examination of the CBF-*COR* gene pathway in *sfr* mutants

The sensitive to freezing mutants studied in this investigation (*sfr4*, *sfr5*, *sfr8* and *sfr9)* all resulted from ethyl methanesulfonate (EMS) chemical mutagenesis, which introduces single nucleotide polymorphisms (SNPs) into the genome. For each mutant, one of these SNPs rendered a gene crucial to the freezing tolerance pathway non-functional, as has been shown in previous literature (Warren *et al.* 1996). Although rare, it is possible for two SNPs to be introduced into the same gene; two base substitutions could potentially have an even greater effect on gene expression.

The most well-studied freezing tolerance pathway is that involving the C-repeat binding factors. These transcription factors regulate the cold regulated (*COR)* genes via the dehydration-responsive element/C-repeat (DRE/CRT) present in their promoters (Jaglo-Ottosen *et al.* 1998, Thomashow 2010). When the temperature drops, expression of the *CBF* genes is activated, and the CBF transcription factors activate *COR* genes. This pathway has been proven to be essential to the development of plant freezing tolerance (Stockinger *et al.* 1997, Liu *et al.* 1998, Thomashow 1999). When mutations occur within this pathway the effects on freezing tolerance are devastating, as is shown in the well-studied mutant *sfr6* (Knight *et al.* 1999).

The four mutants studied in this investigation showed a less severe response to freezing when compared with *sfr6* (Warren *et al*. 1996), however a disruption within the CBF pathway was still a potential cause for the phenotype seen in *sfr4*, *sfr5* and *sfr8.* Due to the

importance of the *CBF* pathway to freezing tolerance, measuring the induction of *CBF* genes and two of their downstream *COR* gene targets, protein kinase 2 (*KIN2)* and galactinol synthase 3 (*GOLS3*) was crucial, either to eliminate the CBF pathway as an area of study, or concentrate on it.

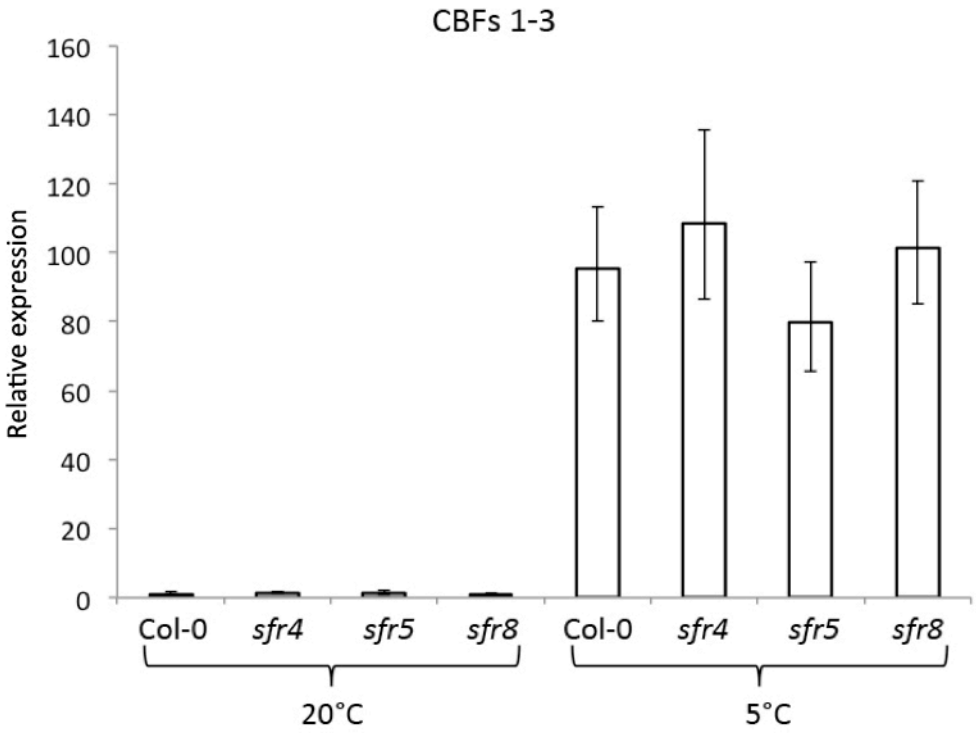


Figure 3.1. Expression levels of CBFs 1-3 *in wild type Col-0,* sfr4, sfr5, *and* sfr8 *following two hours of exposure to 20°C (ambient) or 5°C (cold).*

Gene expression was investigated by the use of quantitative real time PCR (qRT-PCR), which measured relative levels of expression of gene transcript when plants had been exposed to low temperature. If transcript levels of each gene were similar in the mutant and Col-0, the CBF-controlled pathway was considered to be functioning correctly in the mutants and could be eliminated as a candidate for the freezing tolerance deficit.

Expression of the *CBF* genes leads to the activation and expression of *COR* genes, what is thought to be the major pathway that leads to freezing tolerance. As a result, primers were designed for qRT-PCR to generically target *CBF* expression. *CBF1*, *CBF2* and *CBF3* all show high levels of similarity to each other, show very similar expression patterns, and have been shown to be induced rapidly after exposure to low positive temperatures (Medina *et al.* 1999).

Negligible levels of *CBF1-3* expression were observed in ambient temperature-treated Col-0 and *sfr* samples alike, but after two hours at 5°C there was an increase in the level of detected *CBF* expression in all samples (figure 3.1). These results were supported by an independent biological repeat (data from biological repeats are not shown). This shows that the expression of *CBF1-3* is unaffected in the *sfr* mutants tested, suggesting that the SNP responsible for the freezing sensitivity of these mutants introduces the freezing tolerance defect downstream of the *CBF*s or in another pathway that leads to freezing tolerance.

3.2. Downstream targets of *CBF* genes

KIN2 is a so-called *COR* gene, the expression of which is activated by binding of the CBF transcription factors to the DRE/CRT element found in the promoter of all *COR* genes (Jaglo-Ottosen *et al.* 1998). Due to this, the rise in *KIN2* expression occurred much later than in the *CBFs*, and as a result the time point chosen for this experiment was six hours (Knight *et al.* 1999).

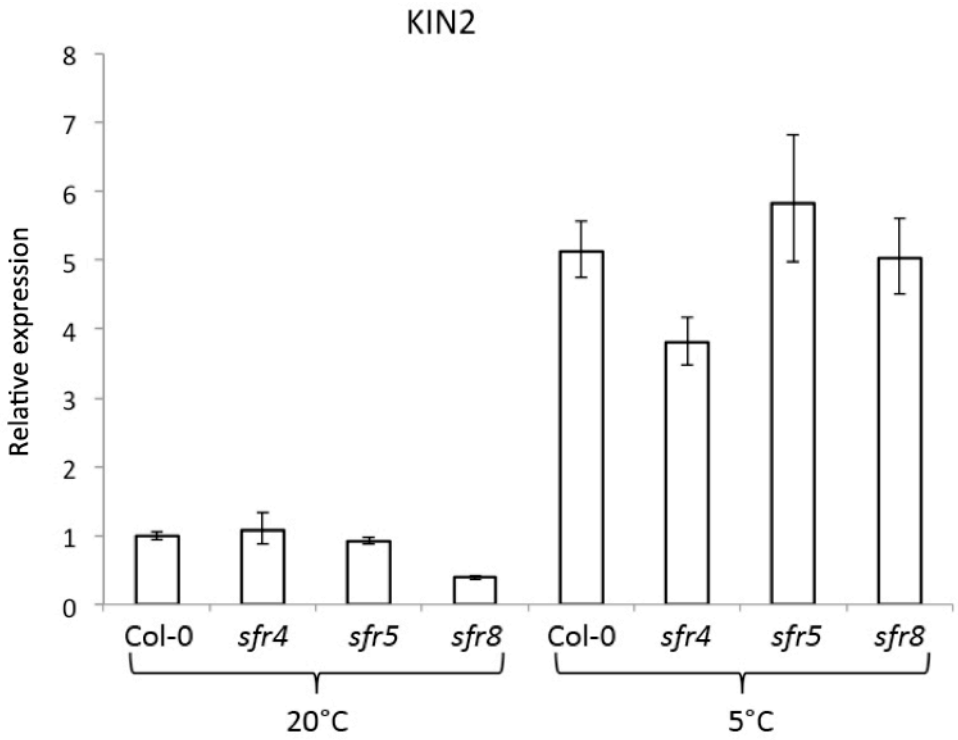


Figure 3.2. Expression levels of KIN2 *in wild type Col-0,* sfr4, sfr5, *and* sfr8 *following six hours of exposure to 20°C (ambient) or 5°C (cold).*

There was evidently a low-level of *KIN2* transcript already present at ambient temperatures (figure 3.2) but, when the temperature was dropped to 5°C, there was an increase in the expression of *KIN2* in all lines tested. *sfr4* induction of *KIN2* was shown to be significantly lower than in Col-0 and the other *sfr* mutants, however an independent biological repeat showed *sfr4* expression levels of *KIN2* in line with the other *sfr* mutants, so this reduction may not be significant.

The expression of another CBF-regulated gene, *GOLS3*, was measured under cold conditions. The COR gene *GOLS3* encodes a protein that protects the plant from oxidative damage when chilling and freezing occur (Nishizawa *et al.* 2008). Like *KIN2*, the time point at which measurements were taken was six hours. As seen in figure 3.3., extremely low levels were present at ambient temperatures; lower than those of *KIN2*. At 5°C, transcript levels

were greatly increased in all of the *sfr* mutants, similar to levels seen in Col-0, suggesting that each of the *sfr* lines tested express *GOLS3* normally. The same result was seen in an independent biological repeat.

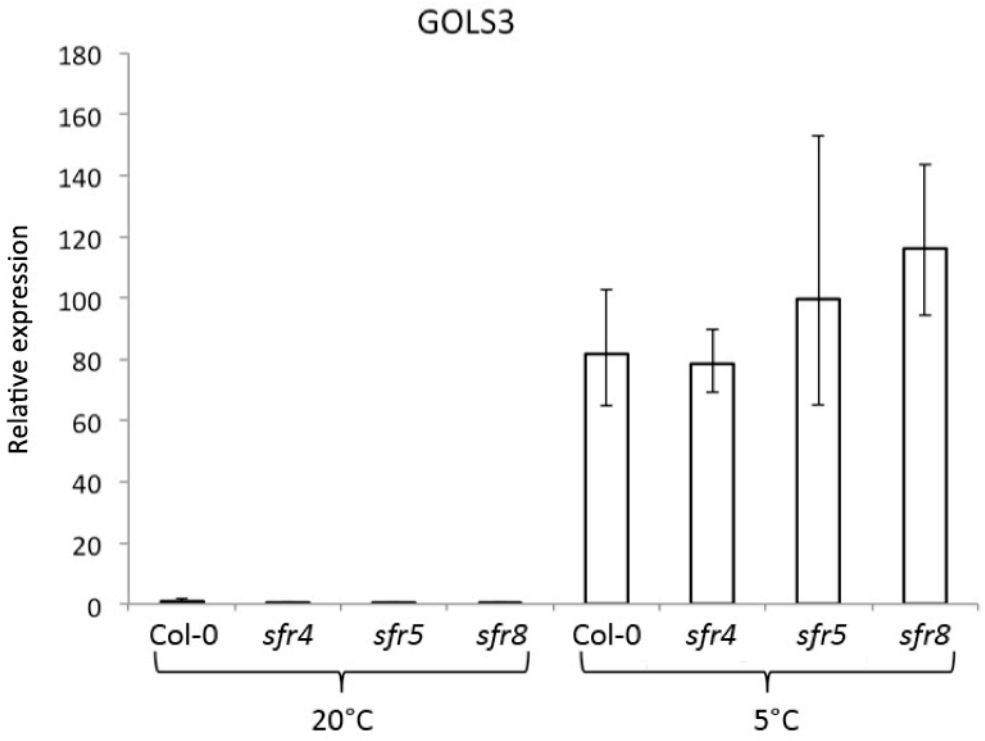


Figure 3.3. Expression levels of GOLS3 *in wild type Col-0,* sfr4, sfr5, *and* sfr8 *following six hours of exposure to 20°C (ambient) or 5°C (cold).*

The results of these assays indicate that the CBF freezing tolerance pathway functions normally in *sfr4*, *sfr5* and *sfr8*. This would suggest that for these *sfr* mutants an alternative pathway might be the cause of their deficit in freezing tolerance. It also supports evidence that a fully functioning CBF pathway alone is insufficient for plants to achieve full freezing tolerance.

Gene transcript levels could not be confirmed for *sfr*9 due to the seeds no longer being viable. The mutant was isolated some years ago and, at the beginning of the study described in this thesis it was discovered that these seeds no longer germinated.

3.3. Next generation sequencing of *sfr* mutant genomic DNA

As the best-studied freezing tolerance pathway had been eliminated as the cause of the *sfr4*, *sfr5* and *sfr8* phenotypes, there remained a large number of other possible factors which could have caused a deficit in freezing tolerance. Mapping intervals had been previously determined for each of the mutations, indicating on which chromosome each of the mutations was found, and a map distance for each of the mutations was determined using known markers (Thorlby *et al.* 1999). Through personal communications with Glenn Thorlby, these mapping intervals were refined and translated into 'Atg' codes, giving a specific region of genes in which the SNP causing the mutation should exist.

Due to the recent increase in accessibility of next generation sequencing technologies, it was possible for full genome sequencing to be carried out for *sfr4, sfr5, sfr8 and sfr9*. Dr. Heather Knight extracted genomic DNA from *sfr4*, *sfr5* and *sfr8* using the Qiagen Plant Mini Kit, and from *sfr9* using the CTAB method. All samples were sent to The Genome Analysis Centre (Norwich, UK) for sequencing via the Illumina method; a NGS technique that utilises reversible terminator dyes to create short 100bp 'reads' of the genome.

These reads were assembled onto the reference TAIR 10 genome (Lamesch *et al.* 2012), and SNP differences between the two genomes were noted. From this, SNPs that differed from

Col-0 could be isolated for each mutant and SNPs that occurred within the gene interval for each mutant were recorded.

3.3.1. Analysis methods

The initial analysis method that was applied to the data involved uploading raw NGS data files to the Galaxy (Hillman-Jackson *et al.* 2002) server (methods section 2.11.2), where they were automatically mapped using Bowtie (Langmead *et al.* 2009) on default settings; henceforth referred to as the Galaxy method. The output file for this method was a .bam file, which was viewable on the IGV software (Thorvaldsdóttir *et al.* 2012). This method was extremely user-friendly and required little prior knowledge of bioinformatics. The computing power required was low, and mapping the Illumina reads to the reference genome could be completed using a mid-level desktop computer.

A second method known as the command line method was undertaken by collaborators at the University of Liverpool (Anthony Hall and Laura Gardiner) who are specialists in the analysis of bioinformatics data. This method involved the use of command line bioinformatics programs, (BWA Burrows-Wheeler Aligner) (Li and Durbin 2009), SAMtools (Sequence Alignment/Map) (Li *et al.* 2009), GATK (Genome Analysis Toolkit) (McKenna *et al.* 2010, DePristo *et al.* 2011) and the 'awk' command. This method required access to powerful supercomputers, and a high level of computer programming knowledge was required to execute the commands needed to analyse bioinformatics data in this way. When both the command line method and the Galaxy method yielded results on the same dataset, direct comparisons between the two methods were drawn in terms of how well each method identified SNPs.

3.3.2. Identification of SNPs

Using the Galaxy method, the raw sequence data were scanned in IGV within the mapping intervals (Thorlby *et al.* 1999), which were further refined via personal communications with Glenn Thorlby before the start of this investigation. When considering whether to investigate a SNP further as a possible cause of freezing sensitivity, the following criteria were applied; initially the coding region was searched for SNPs, however if there were no SNPs within the coding region for a given mapping interval, the promoter/3' UTR was considered. Further to this, the SNP had to appear in at least 70% of reads available for that nucleotide.

The command line method did not require manual identification of SNPs as the selection criteria were part of the process, and the resultant output was SNP data for the whole genome sequence. A rating system listed them as likely to be homozygous or heterozygous, with heterozygous SNPs occurring in close to 50% of reads. Only SNPs within the mapping interval were considered as potential candidates. Some of these SNPs were not found in the coding sequence, and these were discarded as they were considered to be the less likely cause of the phenotype. This preference for SNPs in the coding sequence is a criterion that other groups have also used (Uchida *et al.* 2011).

3.3.3. Data quality and how this relates to mapping

For the four mutant Arabidopsis genomes tested, the mapped genome data varied greatly in quality. The level of mapping to a reference genome was extremely important; if a sample mapped 'poorly', it gave a low level of genome coverage; some areas were not replicated many times, and some areas were not sequenced at all.

All SNPs found *in silico* were also verified in the DNA by PCR. SNPs that are listed as 'verified' in the DNA in tables 3.1 and 3.2 were proven to exist in the genomic DNA by single read ABI sequencing of a PCR amplified amplicon spanning the SNP. The results of these sequence data were then compared to the known reference sequence using NCBI blast, and if the mutation was present, the SNP was investigated further.

3.4. Sequencing data

3.4.1. *sfr4*

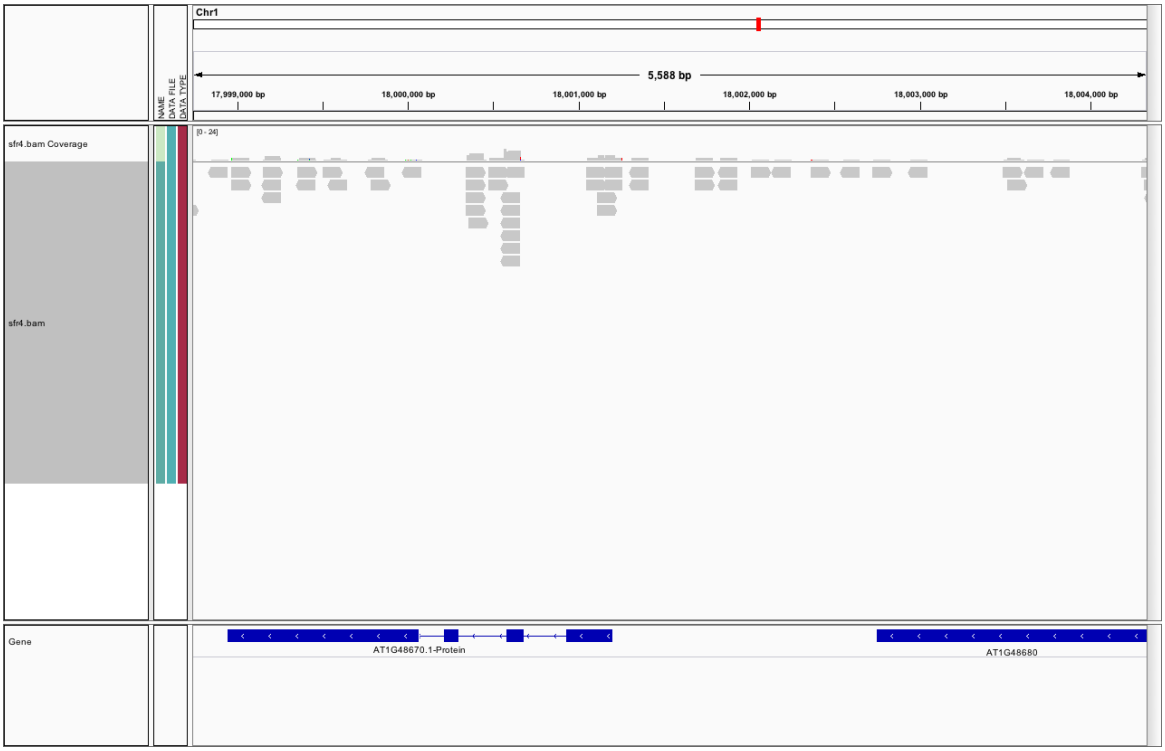


Figure 3.4. Screenshot of IGV showing a representative level of coverage seen in the original sfr4 *data. Each row is a level of sequence, and each bar is a paired-end read.*

The original Illumina run for *sfr4* yielded extremely low quality data which did not map well with the Galaxy method; the number of reads was low and there were large gaps between reads (figure 3.4). No confidence could be given to SNPs found in these data. The Illumina sequencing was repeated and, as a result, the command line was not using the original *sfr4* dataset. The genomic DNA used for the second sequencing attempt was extracted using a different method to that used previously, a modified version of the CTAB method (section 2.8.1).

The results from the second round of sequencing showed a much higher level of coverage for *sfr4*; software used to map the data via the command line method indicated that *sfr4*

had an average coverage of approximately x100 across the entire genome. Despite this, none of the command line-generated SNPs corresponded to the mapping region (At1g48640 – At1g50200). Even when presented with a larger interval (At1g42460 – At1g50200) that, based on the mapping results, almost definitely contained the *sfr4* mutation, still no SNPs were found. This sequencing data was made available too late to be mapped using the Galaxy method, and as a result there are no SNPs for this mutant.

3.4.2. *sfr5*

Similarly to *sfr4*, the *sfr5* data were of insufficient quality to work well with the Galaxy method; this sequencing run gave an average coverage of 10-15x across the genome, but when these data were viewed on the Galaxy platform, there were areas within the mapping region that showed lower coverage than this. The command line method mapped these data to the reference genome with more success than the Galaxy method, and as a result did give SNP data for further analysis, however this meant that no comparison could be made between the two methods for this mutant.

The SNPs found via the command line method were compared to the known gene interval (At1g13490 - At1g17290) and several SNPs were found to exist within the interval, as shown in table 3.1. The system used in the command line method ranked the likelihood of SNPs to represent 'homozygous' or 'heterozygous' mutations in the genome; those ranked as heterozygous were expected to occur in 50% of the reads, and homozygous SNPs as close to 100%. Each SNP was also ranked on a scale from 'least convincing' to 'highly convincing' in terms of how likely it was to exist within the genome, rather than being a product of mapping or sequencing errors. All SNPs within the mapping interval that were located in the

coding regions of genes (bar the SNP listed as 'least convincing') were checked via PCR and sequencing to see if they were present in the DNA of *sfr5.* All of the SNPs listed as 'less convincing' were proven to be a product of sequencing and/or mapping errors, whereas the 'highly convincing' heterozygotes in genes were proven to exist.

Table 3.1. SNPs found via the command line method for sfr5*. The 'Likelihood of SNP' column represents how likely it is that this SNP will appear in the genomic DNA of the mutant. All SNPs listed as 'less convincing' were proven to be a product of the sequencing of mapping processes; as a result the SNP listed as 'Least convincing' was not included.*

Position of SNP	Gene	Original base	Mutated base	Likelihood of SNP	Likely to be	Presence of mutation
Chr1:4644027	At1g13570	G	T	Less convincing	Heterozygote	Tested; not found in DNA
Chr1:4834566	At1g14120	T	A	Less convincing	Heterozygote	Tested; not found in DNA
Chr1:5205941	-	T	G	Highly convincing	Homozygote	Not tested in DNA
Chr1:5300535	**At1g15410**	**A**	**G**	**Highly convincing**	**Homozygote**	**Tested; found in DNA**
Chr1:5402039	**At1g15690**	**A**	**G**	**Highly convincing**	**Homozygote**	**Tested; found in DNA**
Chr1:5519332	At1g16110	G	C	Less convincing	Heterozygote	Tested; not found in DNA
Chr1:5519333	At1g16110	G	A	Less convincing	Heterozygote	Tested; not found in DNA
Chr1:5807864	At1g16980	C	A	Least convincing	Heterozygote	Not tested in DNA
Chr1:5819658	-	A	C	Highly convincing	Homozygote	Not tested in DNA
Chr1:5872831	At1g17180	A	C	Less convincing	Heterozygote	Tested; not found in DNA

3.4.3. *sfr8*

The data for *sfr8* mapped well with the Galaxy method. Bowtie aligned reads to a large percentage of the genome, with very few areas showing gaps between reads or low numbers of reads over a certain area. These data were also mapped using the command line method, which predicted an average of 20x coverage across the genome.

The gene interval in which the SNP causing *sfr8* was proposed to exist ran from At3g48750 to At3g57270. In table 3.2 all SNPs found for *sfr8* using both analysis methods are listed, indicating which method(s) identified the SNP. The direct comparison of these two methods on the same data yielded results as to how well the two methods succeed in identifying SNPs in this type of data, and whether the same SNPs were identified. This comparison also included verifying if the SNP was present in the DNA of the mutant plant, as this gave real proof as to whether a method had successfully detected a 'real' SNP. Four SNPs in three different genes were found via both methods, with the majority of the SNPs found via the Galaxy method falling below the threshold coverage levels used by the command line method. One of these SNPs, in At3g56590, was found too late to be worked on further, and as result is a suggestion for further work.

Table 3.2. SNP data for sfr8*, identifying which SNPs found via the Galaxy method, were also found by the command line method. No SNPs were found by the command line method alone. In the gene column, * indicates that the mutation was not found in a gene, but the gene recorded is the one it is closest to. **indicates that a mutation was found within multiple copies of the 'same' read (see section 2.11.4.).*

Position of SNP	Gene	Original base	Mutated base	% reads with mutation, (total no.)	Found by command line method?	Presence of mutation
Chr3:18110990	-	T	G	100 (4)	No	Not tested in DNA
Chr3:18544327	-	G	T	100 (5)	No	Not tested in DNA
Chr3:18544328	-	T	C	100 (3)	No	Not tested in DNA
Chr3:18684521	-	T	C	100 (2)	No	Not tested in DNA
Chr3:18715665	-	C	T	100 (4) *	No	Not tested in DNA
Chr3:18738148	-	G	T	100 (9)	No	Not tested in DNA
Chr3:18841182	AT3G50700	C	T	100 (3)**	No	Not tested in DNA
Chr3:18841183	AT3G50700	A	T	100 (3)*	No	Not tested in DNA
Chr3: 18920586	**AT3G50910**	**C**	**T**	**100 (16)**	**Yes**	**Proven in DNA**
Chr3: 19007726	**AT3G51160**	**G**	**A**	**83 (6)**	**No**	**Proven in DNA**
Chr3:19206875	AT3G51780	A	T	100 (10)	Yes	Tested; not found in DNA
Chr3:19206876	AT3G51780	A	T	100 (10)	Yes	Tested; not found in DNA
Chr3:20648053	AT3G55650	C	T	78 (9)	No	Not tested; sense mutation
Chr3:20795011	**AT3G56040**	**A**	**T**	**100 (9)**	**Yes**	**Proven in DNA**
Chr3:20795012	**AT3G56040**	**T**	**C**	**100 (8)**	**Yes**	**Proven in DNA**
Chr3:19082704	-	C	T	75 (4)	No	Not tested in DNA
Chr3:19437232	-	T	C	100 (21)	No	Not tested in DNA
Chr3:19778523	-	T	C	100 (4)	No	Not tested in DNA
Chr3:20852578	AT3G56210 (3'UTR)	C	G	83 (6)	No	Not tested in DNA
Chr3:20966136	AT3G56590	C	T	100 (25)	Yes	Not tested in DNA

3.4.4. *sfr9*

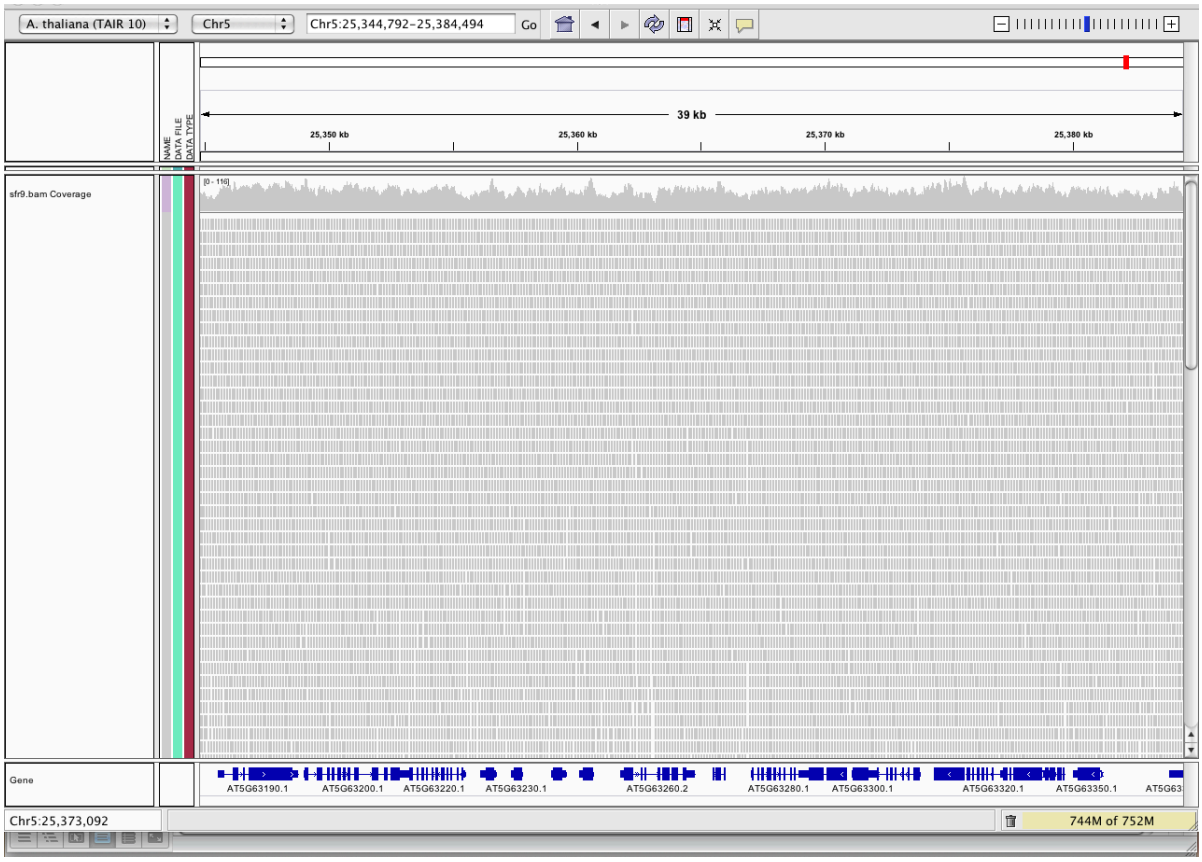


Figure 3.5. Screenshot of IGV showing a representative level of coverage seen for sfr9 Illumina sequence data. Each row is a level of sequence, and each bar is a paired-end read. Shown in the image is only a fraction of the genome coverage; this extended almost three times beyond what is seen.

Like *sfr8*, *sfr9* mapped well using both methods (figure 3.5), allowing direct comparisons between the two. Two intervals were supplied; an extremely refined interval which was not certain to contain the gene (At5g62730 – At5g63640) and a larger interval, in which there was much more confidence. The larger interval ranged from gene At5g61850 - At5g64470.

Coverage was excellent for this mutant, with complete coverage of the mapping interval; average coverage of the genome was estimated at 70x. However only one SNP was found

within the region using Galaxy; none were found using command line method. This one candidate was in the potential promoter region of At5g62680, glucosinolate transporter 2 (*GTR2*), which is involved in the accumulation glucosinolates in seeds (Nour-Eldin *et al.* 2012) and has been reported to be induced by sucrose (Lejay *et al.* 2008).

GTTCAAGAGGCTTTCTCTCCATTTGTTTGTCTATATTTTTGGCTATAATTATTACTACAAACAAAAAAAGAAGT
TGTCGTATACTTTGTAGTATCTTTATTTTCTTGTGTGTTTTGTTTAGTTTCAGAGACAAATGATGACTATTTTCT
TCATCTTAGAGCTCTCTCTATTTATAGTGGTAAGAAAGAAAGCAAGAGGATGGTGGAGATGTCGGAATCTTGACA
AAGTGGTAATTGGTGAATTTGGCCGTGAAAGAGAGCTCTGTTACTGATTTAAATATTCATTAATTTCTTCTTTTT
TTCTTCTTCCAAAATTAAACTTATAAAATCCATTTAGCAAAATCAGTGAAAAATGTAGATTCCATTAATAAGTGG
TGTGGCCCCATCATAACACTCCCTCTTAATAACGGCACTTTCTTTTACGCTCTCCATCCATTTTCTTATTTTCTA
ATCTTATTCTTCTTCTTTCCACTATTTTTGATCTCATAATCTTATATTTTAATTAACTAGTGTAAAACTGACGTT
GACTTAAATGCTGTATGATTTCGAGATCGATTTTATAAAGTTTTTTCCAAGCATTTTAATTAAATATTCAAATAT
CCTTATAATACATTATCATTTTCGAAAGTTACACTGACTACTATGAATG

Figure 3.6. Position of the mutation in the sfr9 *mapping interval within the Arabidopsis genome. Indicated in blue is the start codon (reverse complement). In red is the 5' UTR of the gene* GTR2*. The highlighted base is the base changed by the SNP, from a G to a C.*

The position of the mutation places it some way from the start codon of the gene, however the gene was still investigated as it was potentially close enough to be affecting the promoter region of the gene, and if disruption to the promoter was sufficiently severe, this could be responsible for the phenotype seen in *sfr9*.

3.5. Investigating candidate SNPs

Once the SNPs had been identified within each mapping interval as described above, experiments were conducted to investigate whether or not any of the genes in which the SNPs occurred were linked to freezing tolerance. This analysis involved testing whether or not the gene was cold-responsive, followed by attempting to identify loss-of-function mutants for that gene and finally, testing those loss-of-function mutants for reduced freezing tolerance, in order

to be able to link the gene to the phenotype. In order to be able to test freezing tolerance, an appropriate assay was required, the development of which is described below. Unfortunately, the second set of *sfr4* sequence data was received at the end of this MSc project and therefore *sfr4* could not be subjected to this analysis.

3.5.1. Transcript levels of candidate genes under cold and ambient conditions

Candidate genes (genes that each contained a SNP and were present in the given gene interval for each mutant) were tested for up-regulation under cold conditions in Col-0 seedlings. Up-regulation under cold conditions might indicate a role in cold acclimation, as is seen in the *CBFs*, *KIN2* and *GOLS3* (Medina *et al.* 1999, Thomashow 2010). A gene not being up-regulated in cold would not, however, be discarded as a potential candidate for being linked to freezing sensitivity, as there are examples of genes being involved in the freezing tolerance pathway that are constitutively expressed. The most well-known example is *ICE1* (Inducer of CBF Expression) which regulates the CBFs (Chinnusamy *et al.* 2003).

3.5.2. Identification and validation of insertional mutants in candidate genes

For a SNP to be successfully linked to a freezing sensitive phenotype, further mutant alleles that displayed the same phenotype as the *sfr* mutant needed to be generated for each prospective gene candidate. For each of the candidate genes, T-DNA insert lines were obtained and genotyped as described in the methods, section 2.9.2.1. When homozygous T-DNA insertion lines were isolated, it was vital to check whether the insertion was actually causing any

disruption to the levels of transcript expressed for each candidate gene. qRT-PCR was used to quantify relative transcript levels of the gene.

T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress) was used to determine the insert site, and Primer3Plus (https://www.bioinformatics.nl/primer3plus) was used to design primers to span the predicted insert site (one upstream of the putative insertion site and one downstream) so that, when the primers were used in conjunction with wild type cDNA normal expression of the particular gene would be seen. However, if the insertion disrupted the transcript, extremely reduced or no gene expression would be seen in the putative knockout line. These primers were verified to work efficiently, as described in section 2.10 of materials and methods. If normal wild type transcript levels were measured in the insert line, this line would not be useful in this investigation as expression of the candidate gene would not be impaired.

*Table 3.3. T-DNA insertional mutants and their corresponding gene/*sfr *mutant. The outcome of the mutant (whether it was successful or not) is listed.*

Mutant	Gene	NASC name	Supplied as hom/het?	Confirmed by PCR	How many plants tested (total no.)	Problems encountered	Final status
sfr5	At1g15690	GK596C07.01	Het	Hom	8 (8)	None	Successful
		GK596C07.02	Het	Hom	8 (16)	None	No longer needed due to success of GK596C07.01
		GK596C07.03	Het	Did not need checking as homozygotes already confirmed	-	None	
		GK596C07.04	Het		-	None	
		GK596C07.05	Het		-	None	
		GK596C07.06	Het		-	None	
		GK596C07.07	Het		-	None	
		GK596C07.08	Het		-	None	
		GK596C07.09	Het		-	None	
		GK596C07.10	Het		-	None	
		GK596C07.11	Het		-	None	
		GK596C07.12	Het		-	None	
	At1g15410	None available			-	None	-
sfr8	At3g50910	SALK_074693C	Hom	Hom	Pool of seedlings	None	Successful
		SALK_124555C	Hom	Hom	Pool of seedlings	None	Unsuccessful
		SALK_132810C	Hom	Hom	8 (16)	None	Successful
		SALK_150964	Het	Problem	8 (8)	No T-DNA bands	Unsuccessful
	At3g51160	SALK_027379	Het	Problem	15 (15)	Only one het; subsequently died	Abandoned - turned to NASC-approved *mur1* lines
		SALK_027387	Het	Problem	16 (16)	No T-DNA bands	
		SALK_057153	Het	Het	8 (13)	Plants extremely ill	
	At3g56040	SALK_020654C	Hom	Hom	Pool of seedlings	Extremely poor germination rate	Successful
sfr9	At5g62680	SALK_052178C	Hom	Hom	Pool of seedlings	None	Unsuccessful

3.5.3. Adult plant freezing assay

The *sensitive to freezing* mutants do not recover from damage sustained during freezing, even when subjected to freezing temperatures post-acclimation. This phenotype is demonstrated by a freezing assay, which is consistent with previous findings (Warren *et al.* 1996). Adult plants between four and five weeks of age, of Col-0, *sfr4*, *sfr5* and *sfr8* mutants were subjected to the standard freezing assay used throughout this investigation. The conditions of two weeks at 5°C, 24 hours at -7.5°C, followed by three days of recuperation at 20°C were sufficient to allow Col-0 plants with functional freezing tolerance to recover, while the *sfr* mutants did not (Figure 3.7). When frozen, to counteract edge/insulation effects of the freezing chamber, all plants tested were randomised within trays. Flags that did not interfere with the experiment were used to identify which line each individual plant belonged to.

If plants that contained a T-DNA insert were subjected to these exact assay conditions and were unable to recover, this suggested that the affected by the T-DNA insertion in was linked to freezing tolerance and therefore potentially the same gene that had been affected by the original EMS screen in the *sfr* mutant.

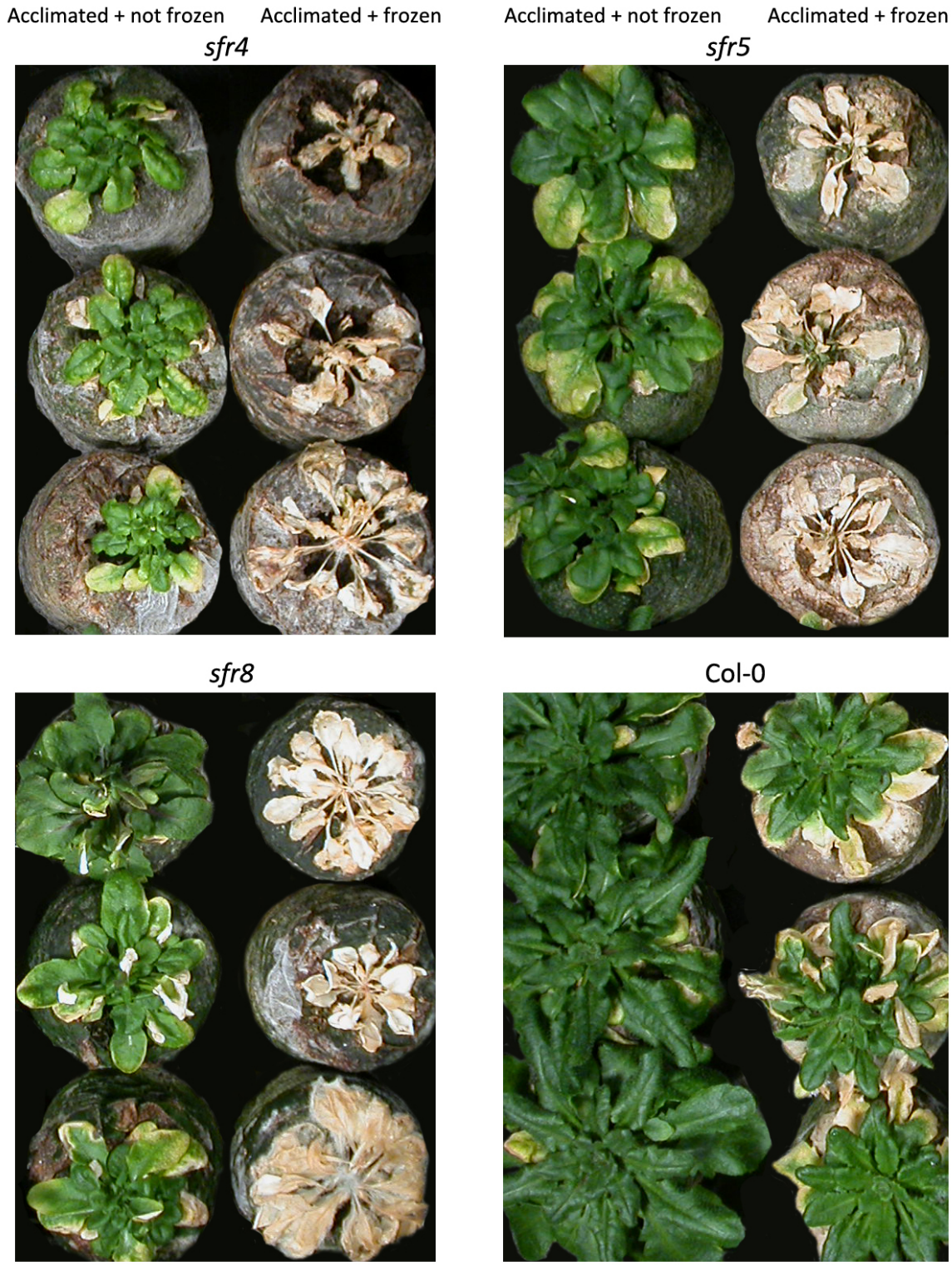


Figure 3.7. A representative sample of a standard freezing assay. All plants were grown under standard short day conditions, and were then transferred to 5°C for two weeks to allow cold acclimation to occur fully. Those marked as 'Acclimated + frozen' were at this point transferred to -7.5°C for 24 hours; those marked 'Acclimated + not frozen' remained at 5°C for this 24-hour period. After the freezing event, all plants were transferred to a 20°C short day growth room to allow them to recover. Photographs were taken three days after transferral to 20°C.

3.6. *sfr4* candidates

No *sfr4* candidates were identified within the specified gene interval and as a result no further work could be carried out on this mutant. For the given gene interval, all of the genes present were inspected to see what they controlled. None of these genes seemed to be directly related to sugar, which was unexpected, as previous work on *sfr4* has suggested that it fails to accumulate sugar during the cold acclimation process (McKown *et al.* 1996, Uemura *et al.* 2003).

3.7. *sfr5* candidates

The command line analysis method resulted in the identification of two candidate genes for the *sfr5* mutation: At1g15690 and At1g15410 (as shown in table 3.1). There were initially other candidates (listed in the table), but these were not found in the DNA of the mutant and as a result were eliminated from the search. At1g15690 encodes AVP1, a H^+ pyrophosphotase which maintains vacuolar pH and controls auxin transport (Li *et al.* 2005). It is known to be involved in the response to water deficit stress (Pasapula *et al.* 2011). Over expression of this H^+ pump has been shown to increase drought and salt tolerance when over-expressed in Arabidopsis. (Gaxiola *et al.* 2001, Li *et al.* 2005, Pasapula *et al.* 2011). Previous work on this gene has shown that the *avp1-1* mutation has an extreme effect on both root and shoot development, rendering the mutant plants much smaller than the Col-0 controls (Li *et al.* 2005). Flowering is also affected; only 30% of *avp1-1* null plants initiated flower development, with none of these developing a full flower, rendering the mutant sterile (Li *et al.* 2005).

sfr5 has never shown any difficulties when flowering, and produces viable seed when the mutation is homozygous. However, the mutant described in this literature is a complete null, whereas *sfr5* is the result of a SNP. A single base change could potentially cause a much smaller change to the resultant protein and, if the mutation occurs some way downstream of the start codon, the protein may be truncated but still functional to a limited degree. The Arabidopsis gene At1g15410 was identified in part of a large study and has not received further work since then. It was predicted to be involved in racemase and epimerase activity, however this has not been biologically verified (Chen *et al.* 2007).

3.7.1. *sfr5* candidate 1: At1g15690 *(AVP1)*

AVP1 was tested for cold-inducibility via qRT PCR but showed no difference in expression after either two hours or six hours of cold treatment, when compared to ambient temperatures. (Figure 3.8). This experiment underwent an independent biological repeat, which supported these results.

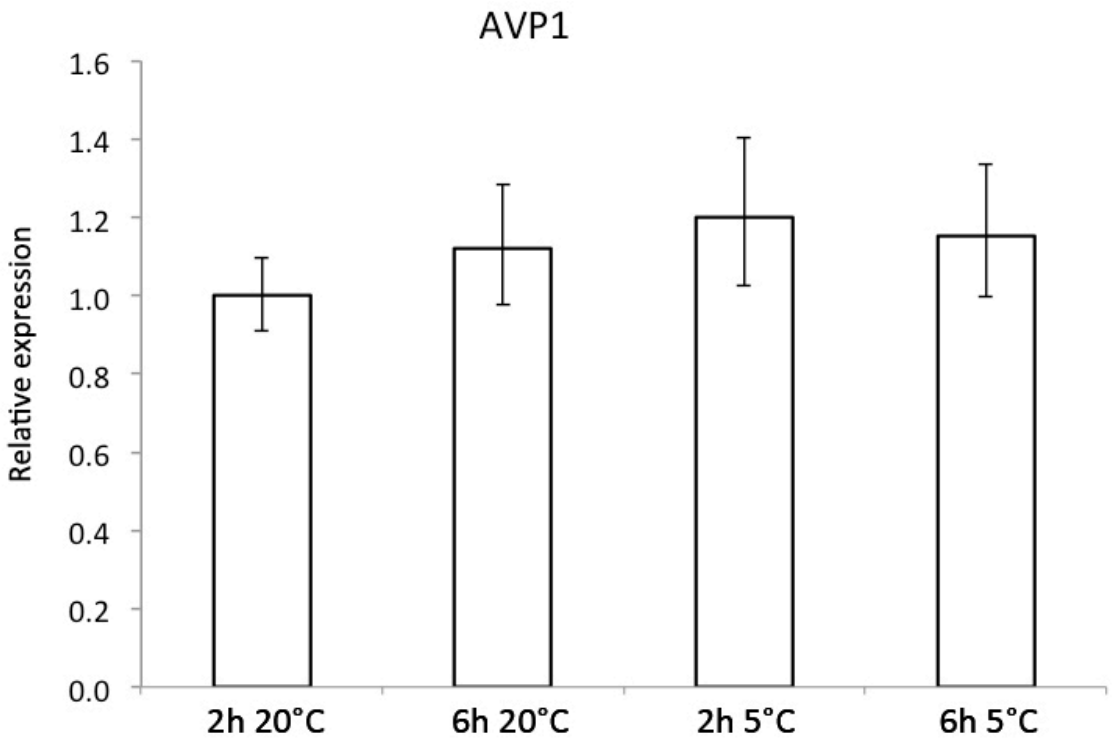


Figure 3.8. Expression levels of AVP1 *in wild type Col-0 after exposure to 20°C (ambient) or 5°C (cold) at two time points, two hours and six hours.*

3.7.2. Insert lines for *AVP1*

No homozygous SALK lines were found for this candidate; however, one heterozygous GABI population (GK_596C07) was identified for *AVP1*. 12 T_3 seed-sets were dispatched; each set was the progeny of a T_2 plant harbouring at least one copy of the T-DNA insert within the gene. One of the T_3 lines was identified as being homozygous, and this was followed up. Seeds were collected from the homozygous individual, and seedlings were grown. These seedlings were once again tested to verify the homozygous status of the mutant before progression to freezing assays and qRT-PCR. As shown in figure 3.9, GK_596C07 showed severely reduced expression of the full-length transcript, and as a result GK_596C07 plants were deemed suitable for the freezing assay. No independent biological repeat were available for this experiment. While not ideal, this was deemed to be acceptable as the

reduced expression of this gene was not expected to be specific to environmental conditions.

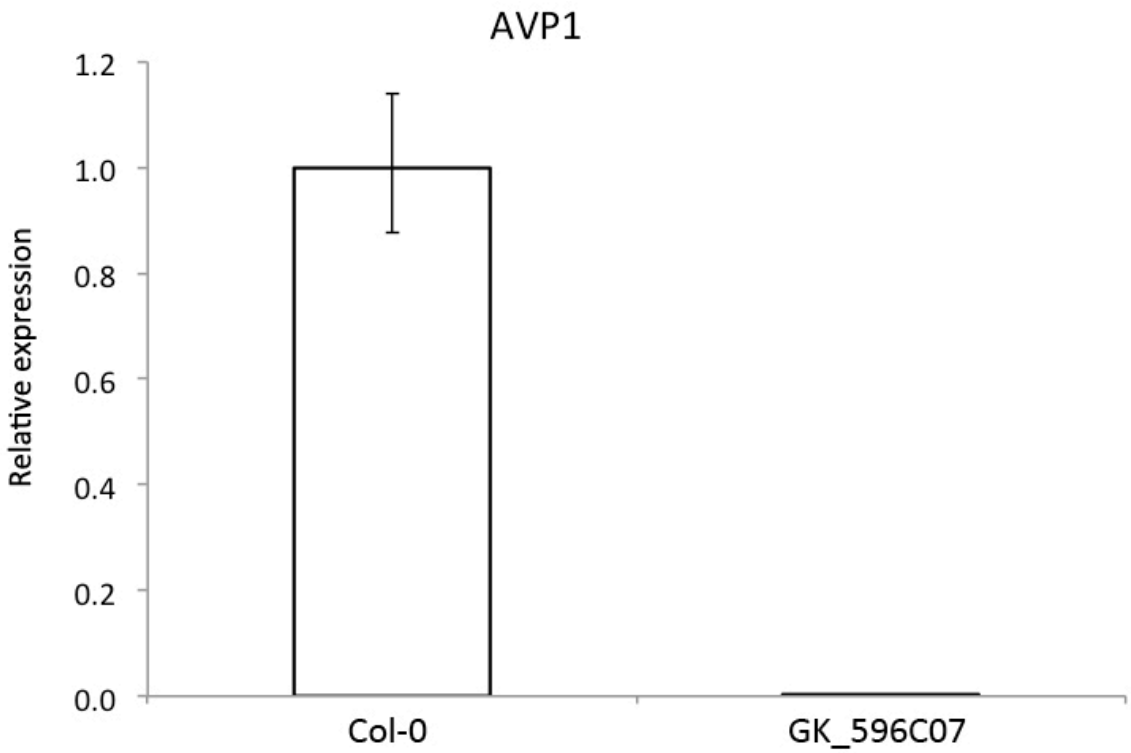


Figure 3.9. Expression levels of AVP1 *in Col-0 and GK_596C07.*

3.7.3. Freezing assay for *AVP1* insert lines

As shown in figure 3.10, the GK_0596C07 plants survived to a much higher level than *sfr5*, and are possibly healthier than the Col-0 plants. As the gene was knocked out completely in the insertional mutant, this would suggest that this gene is not responsible for the *sfr5* mutation.

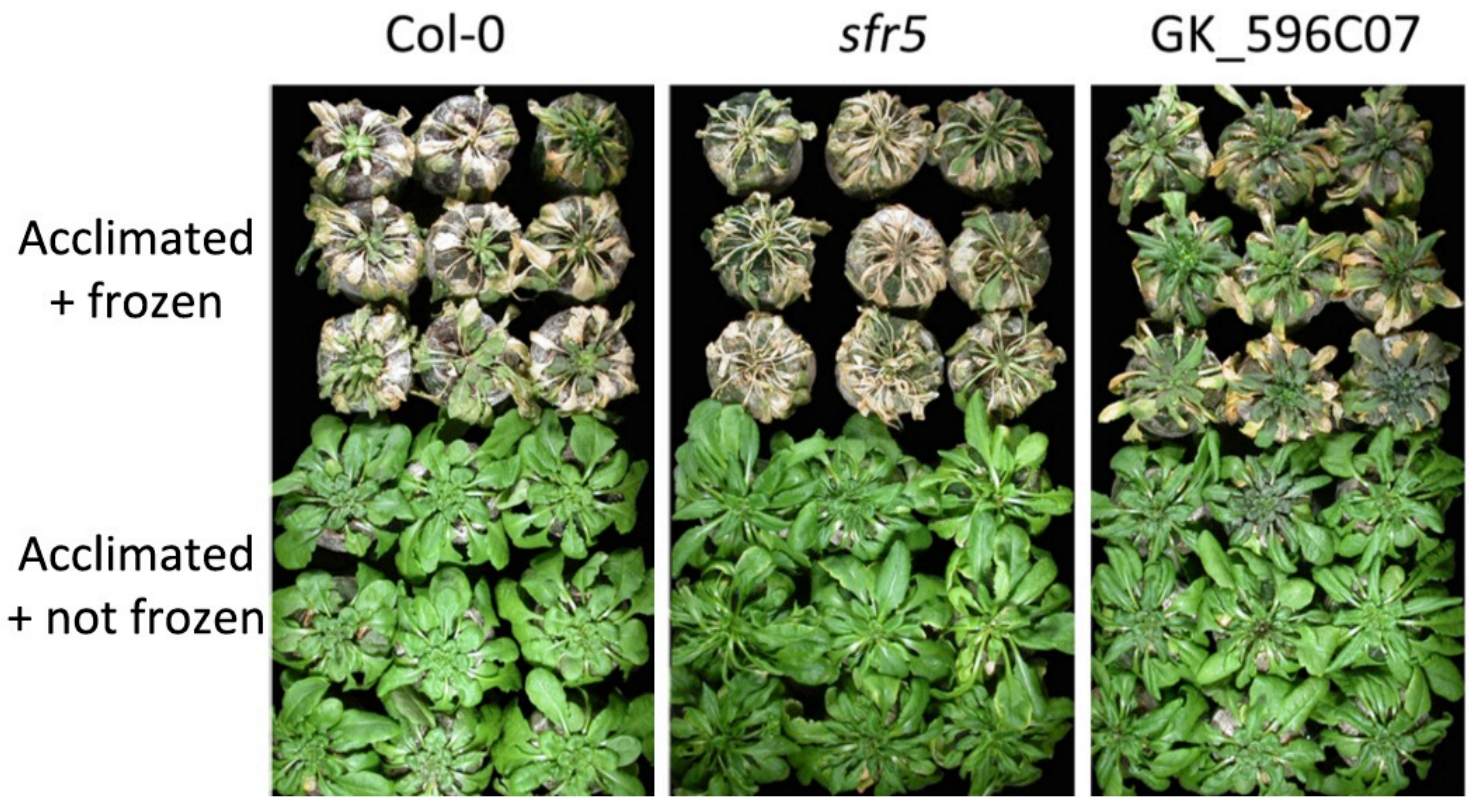


Figure 3.10. Freezing assay conducted on Col-0, sfr5 *and GK_596C07 plants. All plants were grown under standard short day conditions, and were then transferred to 5°C for two weeks. Those marked as 'Acclimated + frozen' were at this point transferred to -7.5°C for approximately 24 hours; those marked as 'Acclimated + not frozen' remained at 5°C for this 24-hour period. After the freezing event, all plants were transferred to a 20°C short day growth room to allow them to recover. Photographs were taken three days after transferral to 20°C.*

3.7.4. *sfr5* candidate 2: At1g15410

At1g15410 was tested for cold inducibility. As shown in figure 3.11, there was no evidence of cold-induction after two or six hours at 5°C. There was, however, a difference in expression between the two time points, suggesting that this gene may be either circadian or diurnally regulated. An independent biological repeat also displayed this time-regulated pattern of expression.

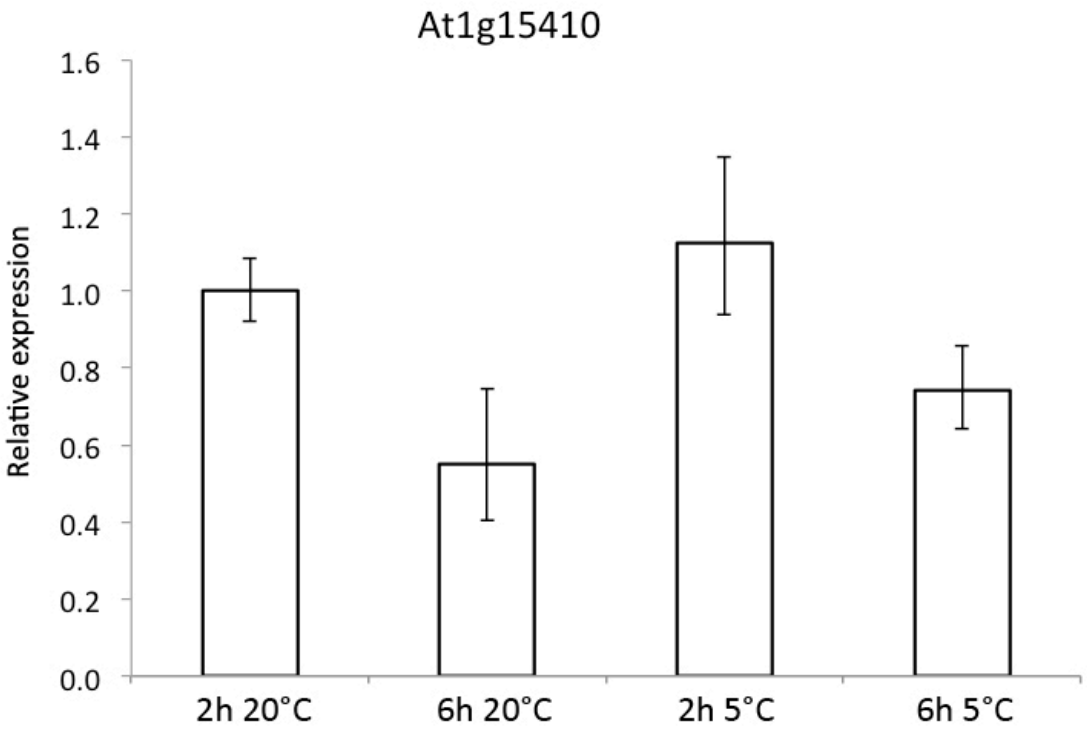


Figure 3.11. Expression levels of At1g15410 in wild type Col-0 after exposure to 20°C (ambient) or 5°C (cold) at two time points, two hours and six hours.

3.7.5. Insert lines for At1g15410

No suitable insertional mutants were available for this candidate gene. Due to time constraints, no further investigations into this candidate could be conducted. Had time permitted, an RNAi knockdown construct would have been created, using a pHELLSGATE vector. Col-0 plants would have been transformed with a plasmid containing the knocked-down gene, and expression levels would be tested.

3.8. *sfr8* candidates

Three candidate genes were initially identified for *sfr8* using the Galaxy method; At3g50910 is an unknown protein that has received little work, At3g51160, Murus 1 (*MUR1*) encodes an enzyme that catalyzes the first step in the de novo synthesis of GDP-L-fucose, mutants of which have been shown to display a distinct phenotype; rounded leaves, weak inflorescences, and reduced tolerance of drought. At3g56040 encodes UDP-glucose pyrophosphorylase 3 (UGP3), which is required for biosynthesis of sulpholipids, a constituent part of photosynthetic membranes in the chloroplast (Okazaki *et al.* 2009).

3.8.1. *sfr8* candidate 1: At3g50910

qRT-PCR was used to measure the expression of At3g50910 and indicated that this gene was slightly up-regulated in cold after both two hours and six hours. However this result is less accurate for two hours, due to the error bars overlapping. The fact that this gene is mildly up-regulated by low temperature may be consistent with a role in low temperature tolerance. Extremely similar results were seen for an independent biological repeat, which could neither confirm nor deny these findings.

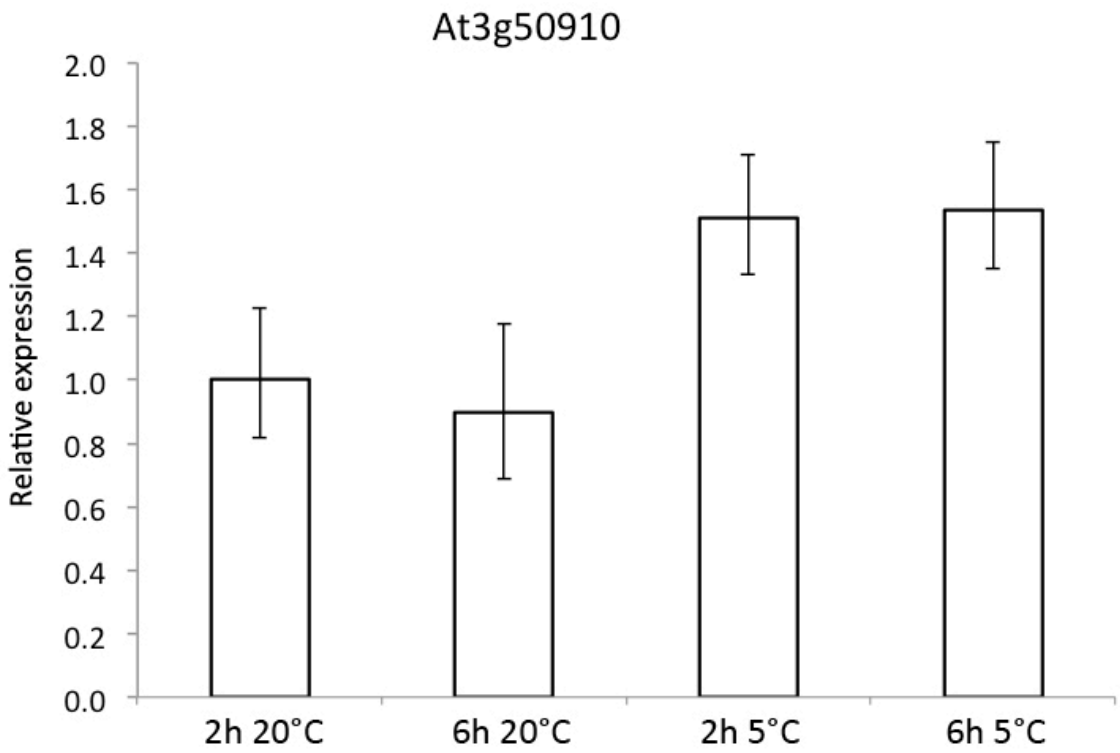


Figure 3.12. Expression levels of At3g50910 in wild type Col-0 after exposure to 20°C (ambient) or 5°C (cold) at two time points, two hours and six hours.

3.8.2 Insert lines for At3g50910

The three homozygous SALK lines (SALK_074693C, SALK_124555C, SALK_132810C) for the gene At3g50910 were all successfully proven via PCR to be homozygotes. One heterozygous line was also obtained, but repeated attempts at genotyping failed to demonstrate a T-DNA insertion in the gene. As a result, this line was discarded due to there being three confirmed homozygotes available for this gene. The three remaining SALK lines were tested to verify whether the T-DNA insertion reduced the expression of the gene targeted. For the first two homozygous SALK lines (SALK_074693C and SALK_124555C) the insertion sites were so close together that the same set of primers were used to check expression of the transcript in both lines.

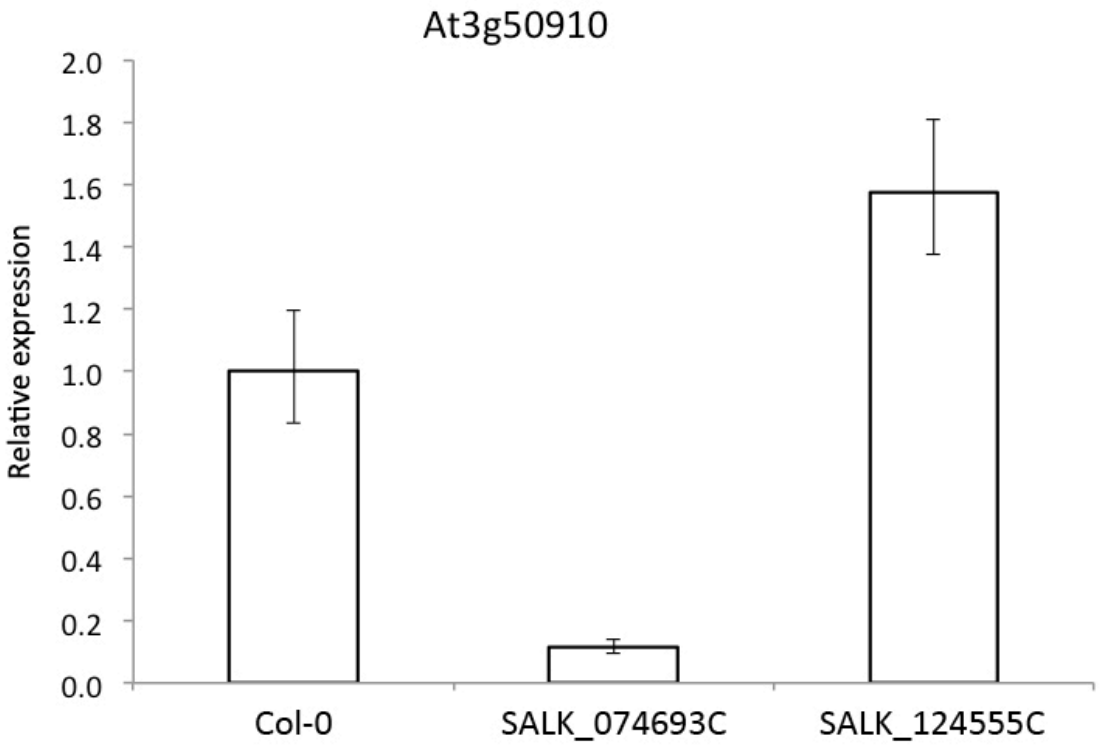


Figure 3.13. Expression levels of At3g5910 in Col-0, SALK_074693C and SALK_124555C.

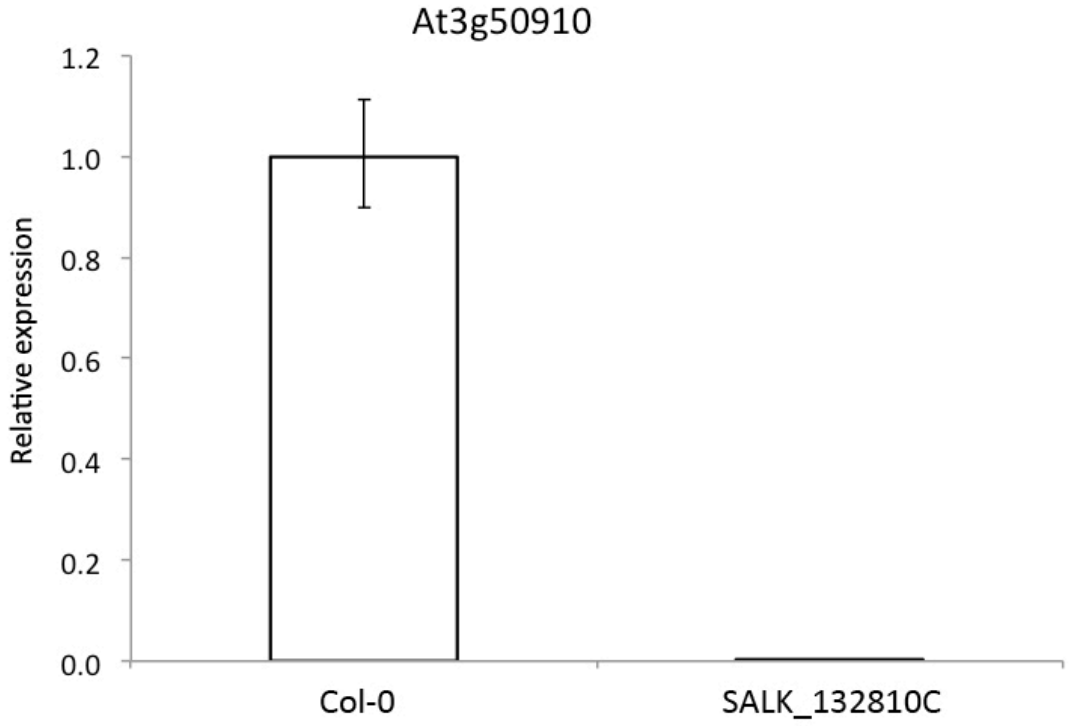


Figure 3.14. Expression levels of At3g5910 in Col-0 and SALK_132810C.

While SALK_074693C shows reduced expression of At3g50910 compared to the Col-0 sample, SALK_124555C actually expressed the gene more effectively than the wild type, figure 3.13, which is not uncommon in this sort of insert line; the 35S promoter that is present in the T-DNA insert can result in over-expression of the Col-0 gene (Ülker *et al.* 2008). SALK_074693C was tested in the freezing assay as expression is reduced. SALK_124555C, however, was not included in the freezing assay. There is no doubt that SALK_132810C (figure 3.14) displays severely reduced gene expression in comparison to the Col-0 control. This sample was confidently included in freezing assays as a knockout mutant for this gene. However, it must be noted that these experiments were completed only once and no biological repeats are available, resulting in this being a suggestion for further work.

3.8.3. Freezing assay for At3g50910 insert lines

Twenty plants for each of the two confirmed homozygous SALK lines for At3g50910 (SALK_074693C and SALK_132810C) were grown up to five weeks, and subjected to the freezing test protocol. The plants were allowed to recover for four days and were then photographed. The recovery of these plants was compared to that seen for *sfr8* (figure 3.15) and the Col-0 control with which they were grown; if any of the SALK lines showed a loss of freezing tolerance, similar to the original EMS mutant, this constituted evidence of linkage. Both SALK lines for this gene candidate, At3g50910, showed recovery levels close to that of the Col-0 (approximately 50%), which would suggest that this gene is not responsible for the freezing sensitivity seen in *sfr8.*

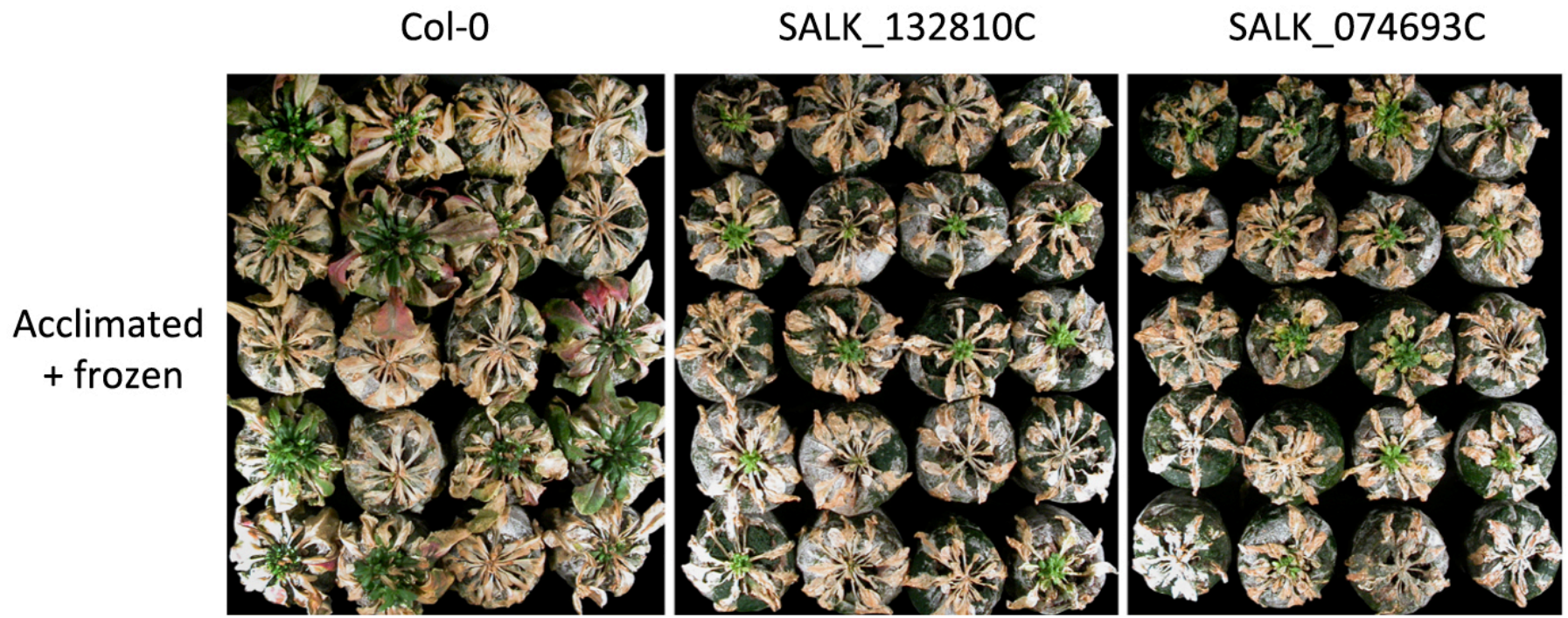


Figure 3.15. Freezing assay conducted on Col-0, SALK_132810C, and SALK_074693C plants. All plants were grown under standard short day conditions, and were then transferred to 5°C for two weeks. Those marked as 'Acclimated + frozen' were at this point transferred to -7.5°C for approximately 24 hours; those marked as 'Acclimated + not frozen' remained at 5°C for this 24-hour period. After the freezing event, all plants were transferred to a 20°C short day growth room to allow them to recover. Photographs were taken three days after transferral to 20°C.

3.8.4. *sfr8* candidate 2: At3g51160 (*MUR1*)

MUR1 was tested to verify whether it was cold inducible. As seen in figure 3.16 it is unlikely that *MUR1* is cold inducible, however it is possible that there is some circadian regulation occurring due to both six-hour time points show reduced levels of transcript compared to the two-hour. A similar pattern was seen in an independent biological repeat.

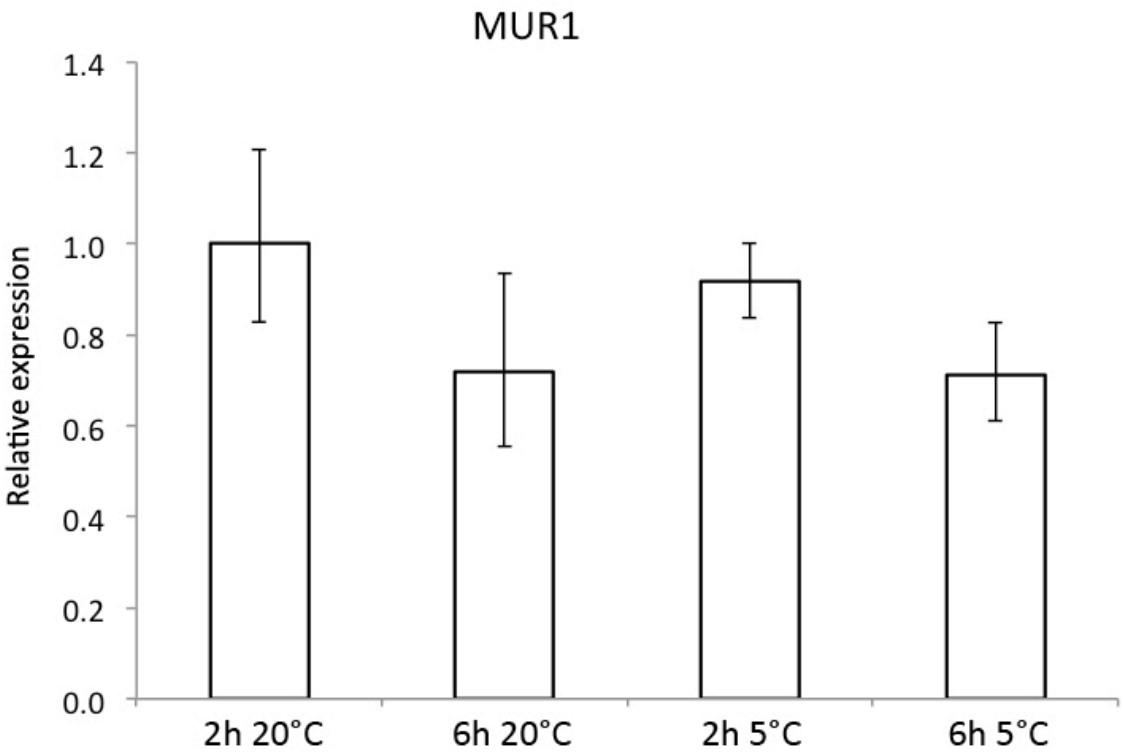


Figure 3.16. Expression levels of MUR1 *in wild type Col-0 after exposure to 20°C (ambient) or 5°C (cold) at two time points, two hours and six hours.*

3.8.5. Insert lines for *MUR1*

Three heterozygous SALK lines were available for the gene At3g51160. Genotyping of these proved difficult; for SALK_027379 16 plants were individually genotyped, and all plants showed an undisrupted Col-0 band and only one showed a T-DNA band (indicating it was a heterozygote). From a second seed batch the results were more promising; two

heterozygotes were identified, and one putative homozygote. This homozygote was grown up but was consistently unhealthy and subsequently died.

Genotyping the heterozygous SALK_027387 line was attempted several times, and both the original seed batch and a replacement seed batch failed to produce a T-DNA band, whilst a band indicating an uninterrupted wild type gene was observed for all samples. As a result this SALK line was abandoned.

The third heterozygous SALK_057153 also proved hard to genotype, with no heterozygous or homozygous individuals being discovered from the first batch of 16 plants. From the second, much like SALK_027279, these heterozygous individuals were extremely weak, and as a result no seed was obtained with enough time to investigate this SALK line further.

Work on this candidate gene proved that heterozygous SALK lines were often problematic, as insertions could not always be confirmed in the material sent out by NASC. The homozygous lines proved to be far more useful to this investigation, and in the case of *MUR1*, there were other available EMS mutants lines that had been verified by ABRC.

3.8.6. Available *mur1* mutants

Three *mur1* mutant lines were available from NASC, and these were used in freezing assays. Reduced expression tests were not needed as the lines supplied were created by EMS mutagenesis (and therefore represented single point mutations rather than complete disruption of the transcript) and had been published previously (Bonin *et al.* 1997). *mur1-1*

and *mur1-2* are the most severely affected, showing only 2% of the Col-0 levels of L-fucose. *mur1-3* is less severely affected, showing ~33% of Col-0 levels of L-fucose.

The two most severely affected lines, *mur1-1* and *mur1-2* show the same response as *sfr8* to freezing treatment; 0% survival, while *mur1-3* showed equivalent survival to wild type. The two severely affected mutants which had been suggested to show dwarfism, appear to be a similar size to *sfr8,* while the Col-0 plants are considerably larger. As the reduction in L-fucose is not that great in *mur1-3*, it is extremely likely that *MUR1* is still a candidate for the freezing tolerance deficit in *sfr8.*

3.8.7. Freezing assay for *mur1* mutants

As shown in figure 3.17*, mur1-1, mur1-2* both reacted in the same way as *sfr8*. *mur1-3* did not, however the L-fucose levels in this mutant are much higher than those in *mur1-1* and *mur1-2*.

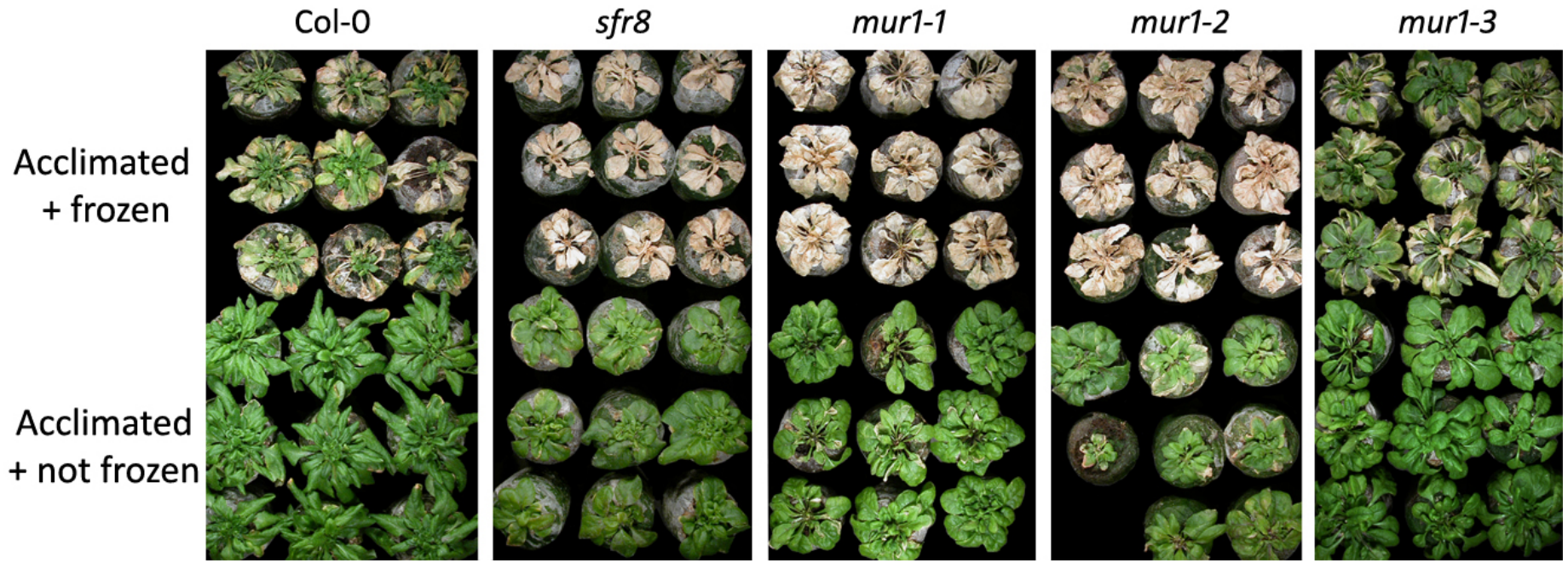


*Figure 3.17. A freezing assay conducted on Col-0, s*fr8, mur1-1, mur1-2, *and* mur1-3 *plants. All plants were grown under standard short day conditions, and were then transferred to 5°C for two weeks. Those marked as 'Acclimated + frozen' were at this point transferred to -7.5°C for approximately 24 hours; those marked as 'Acclimated + not frozen' remained at 5°C for this 24-hour period. After the freezing event, all plants were transferred to a 20°C short day growth room to allow them to recover. Photographs were taken three days after transferral to 20°C.*

3.8.8. *sfr8* candidate 3: At3g56040 (*UGP3*)

UGP3 was tested to verify whether it was cold inducible. As shown in figure 3.18, *UGP3* was shown to display no cold inducibility and no real difference between time points. An independent biological repeat supported this result.

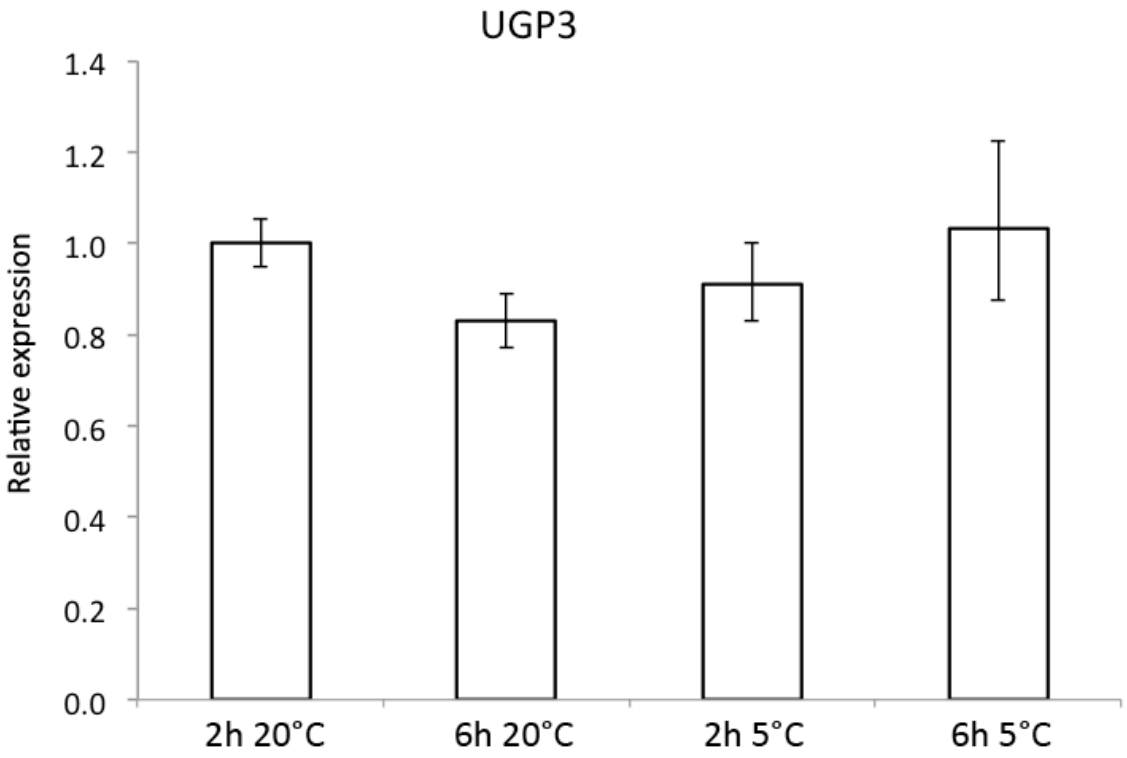


Figure 3.18. Expression levels of UGP3 *in wild type Col-0 after exposure to 20°C (ambient) or 5°C (cold) at two time points, two hours and six hours.*

3.8.9. Insert lines for *UGP3*

One homozygous SALK line, SALK_020654C, was available and from the supplied seed seedlings were grown up. The germination rate of this line was extremely low; from the entire batch very few seedlings survived. Transcript levels of *UGP3* were verified in SALK_020654C and found to very low (Figure 3.19), however this experiment does not have any biological repeats, and would need to be replicated before this work is considered

anything more than preliminary. However, due to these results this mutant was used in subsequent freezing assays.

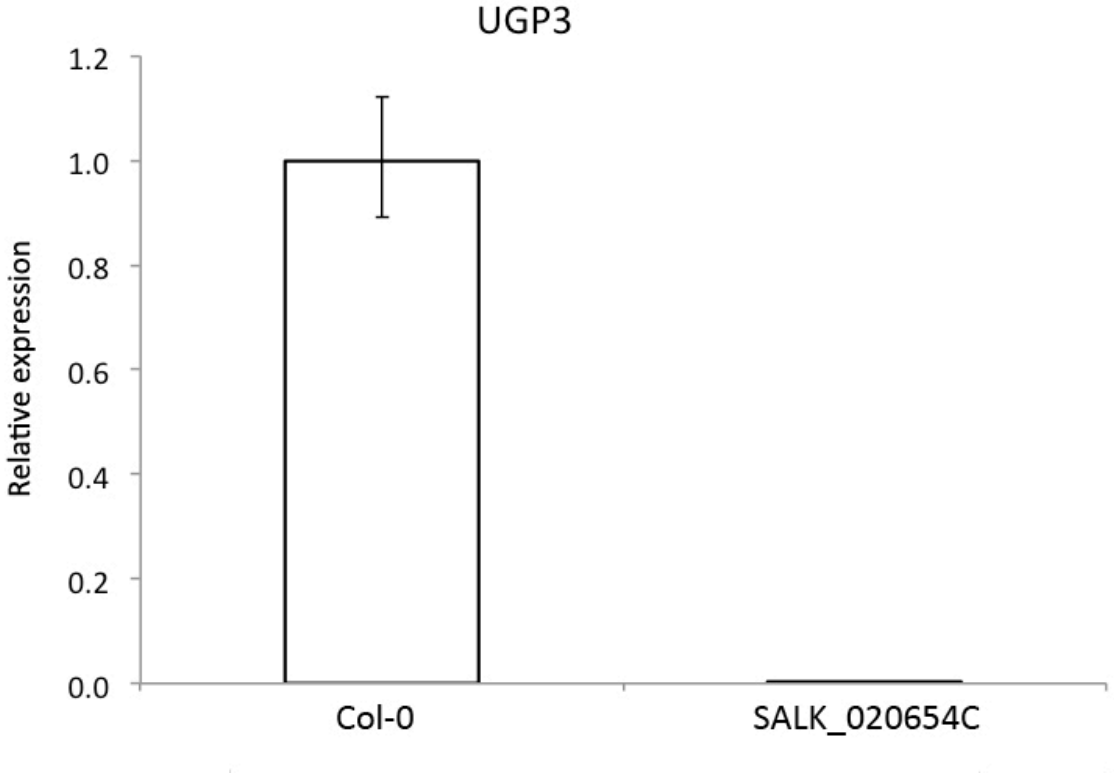


Figure 3.19. Expression levels of At3g5910 in Col-0 and SALK_020654C.

3. 8.10. Freezing assay for *UGP3* insert lines

Due to the low germination rate experienced with this mutant, only one adult plant could be frozen. This plant subsequently died, however there is very little evidence to suggest that this SALK line was or was not tolerant to freezing.

3.9. *sfr9* candidates

3.9.1. *sfr9* candidate 1: At5g62680 *(GTR2)*

Only one candidate gene was considered for this mutant, At5g62680, *GTR2*. This was the gene that corresponded to the only SNP found within the mapping interval for this mutant, and whilst the SNP in the original *sfr9* DNA was not in the coding sequence of the gene, it is theorised that it may be within the promoter region. It is some distance from the 5' UTR, as shown previously (Figure 3.6). As previously mentioned, *sfr9* seeds were no longer viable and did not germinate, so no comparison of freezing tolerance could be conducted between insertion lines and the mutant. The gene was, however, tested for cold induction.

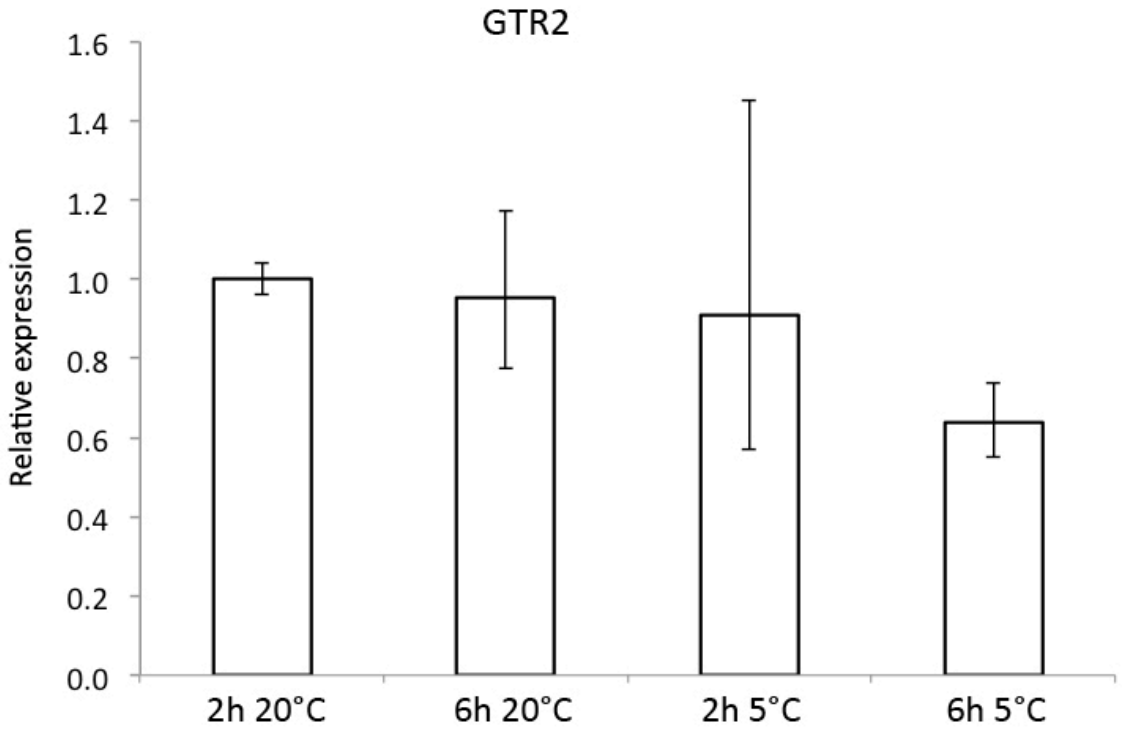


Figure 3.20. Expression levels of GTR2 *in wild type Col-0 after exposure to 20°C (ambient) or 5°C (cold) at two time points, two hours and six hours.*

As shown in figure 3.20, after two hours at 5˚C, there seems to be no difference in transcript levels when compared to the Col-0 sample. After six hours, however, there appears to be slightly reduced transcript levels. This shows that the gene is in no way cold inducible. An independent biological repeat confirmed this to be the case.

3.9.2. Insert lines for *GTR2*

The one homozygous SALK line, SALK_052178C, was grown up and genotyped. The expression of *GTR2* was examined in SALK_052178C. As shown in figure 3.21, the expression of the functional Col-0 gene is higher in the insertional mutant than in the Col-0 sample, resulting in this line being unsuitable for use in freezing tests. No biological repeats are available for this experiment; this result would need to be replicated before this candidate is truly disregarded.

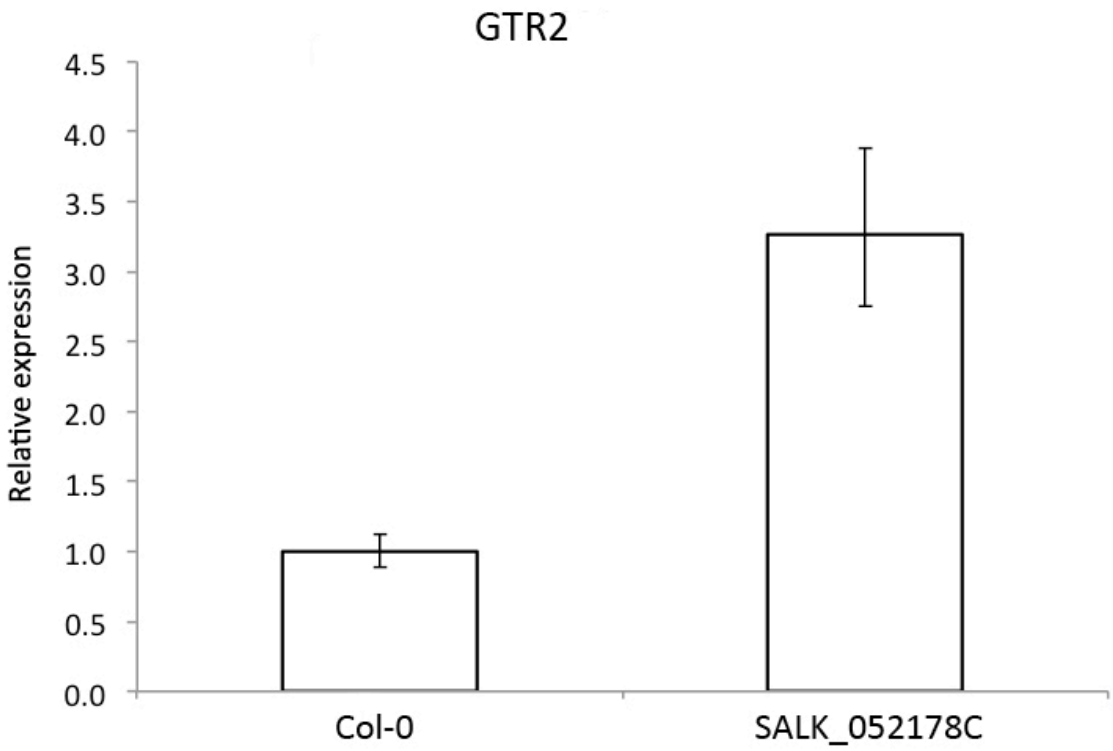


Figure 3.21. Expression levels of GTR2 *in Col-0 and SALK_052178C.*

3.10. Summary of results

Throughout this investigation, the T-DNA insert line available for one candidate for *sfr5, AVP1,* responded in the same way as Col-0 plants. However, it has not yet been ruled out due to phenotypic discrepancies seen between the *avp1* mutant and the T-DNA line, potentially indicating there was a problem with this line that has gone undetected. As a result this candidate warrants further work. Further work on this remaining candidate, At1g15410, is also suggested.

One candidate was successfully eliminated for *sfr8*, At3g50910, through the use of SALK mutants in freezing tests. The survival of these SALKs under freezing conditions would suggest that this gene is not responsible for the freezing tolerance deficit. The second candidate for s*fr8*, *MUR1*, still remains as a strong candidate, but cannot be fully accepted and requires more work. For *UGP3,* the only available homozygous SALK failed to reduce expression of the *UGP3* transcript, and thus no further investigations could be carried out on this mutant.

For the one *sfr9* candidate, the one available homozygous SALK did not produce enough viable seedlings to allow proper freezing testing. As a result, this candidate can neither be favoured nor rejected.

3.11. Further investigations

Little is known about the four sensitive to freezing mutants being investigated; of the four, only *sfr4* has been the subject of further work, and it has been shown that the deficit in freezing tolerance in *sfr4* is due to an inability to accumulate sugars during cold acclimation (McKown *et al.* 1996, Uemura *et al.* 2003).

While not mentioned in the literature, during this investigation it was noticed that *sfr8* flowers earlier than any of the other mutants or Col-0. As a result of this, a number of phenotypic experiments were conducted to learn more about the other effects of the *sfr* mutations. There are other phenotypes (such as susceptibility to drought) which are often linked to freezing tolerance (Kasuga *et al.* 1999). Because of this *sfr4, sfr5* and *sfr8* were tested for alterations in osmotic stress tolerance.

3.11.1. Effect of osmotic stress on seedling emergence

Col-0, *sfr4, sfr5* and *sfr8* seeds were sown onto Petri dishes containing MS media supplemented with differing concentrations of mannitol; 0mM, 200mM, 300mM and 400mM. For each seed line at each concentration, there were five technical replicates. After seven days of growth under standard growth conditions, the number of seedlings that had scored 'yes' or 'no' for radicle emergence was counted under a microscope. The 0mM mannitol plates were used as a baseline level of germination. It was obvious that mannitol affected development, as there was a visible size difference between seedlings grown on MS media and those on media including mannitol (figure 3.22). However the number of seedlings that had undergone radicle emergence was extremely similar for all concentrations of mannitol. Seedlings that had germinated on the highest concentration of

mannitol were very stunted, and several developmental stages behind those grown on just MS agar, but radicle emergence was unchanged.

These results differ from those shown by (Boyce *et. al.* 2003, Wathugala *et al.* 2011), who, when using these conditions, found 400mM mannitol to be enough to reduce radicle emergence to practically nil. However, three biological replicates, each with five technical replicates were conducted for this assay, and the results were consistent between all. As a result the experiment was repeated, with 0mM compared to 500mM mannitol.

Even at 500mM mannitol (figure 3.23), the emergence between the seed lines was not greatly altered (data not shown). Germination on MS media was at around 90-95% for all lines; this dropped to ~20% for all lines at 500mM. This would suggest that none of the *sfr* mutants are affected at the stage at which they first put out a radicle, however this does not eliminate the possibility that adult *sfr* plants are deficient in their ability to tolerate osmotic stress.

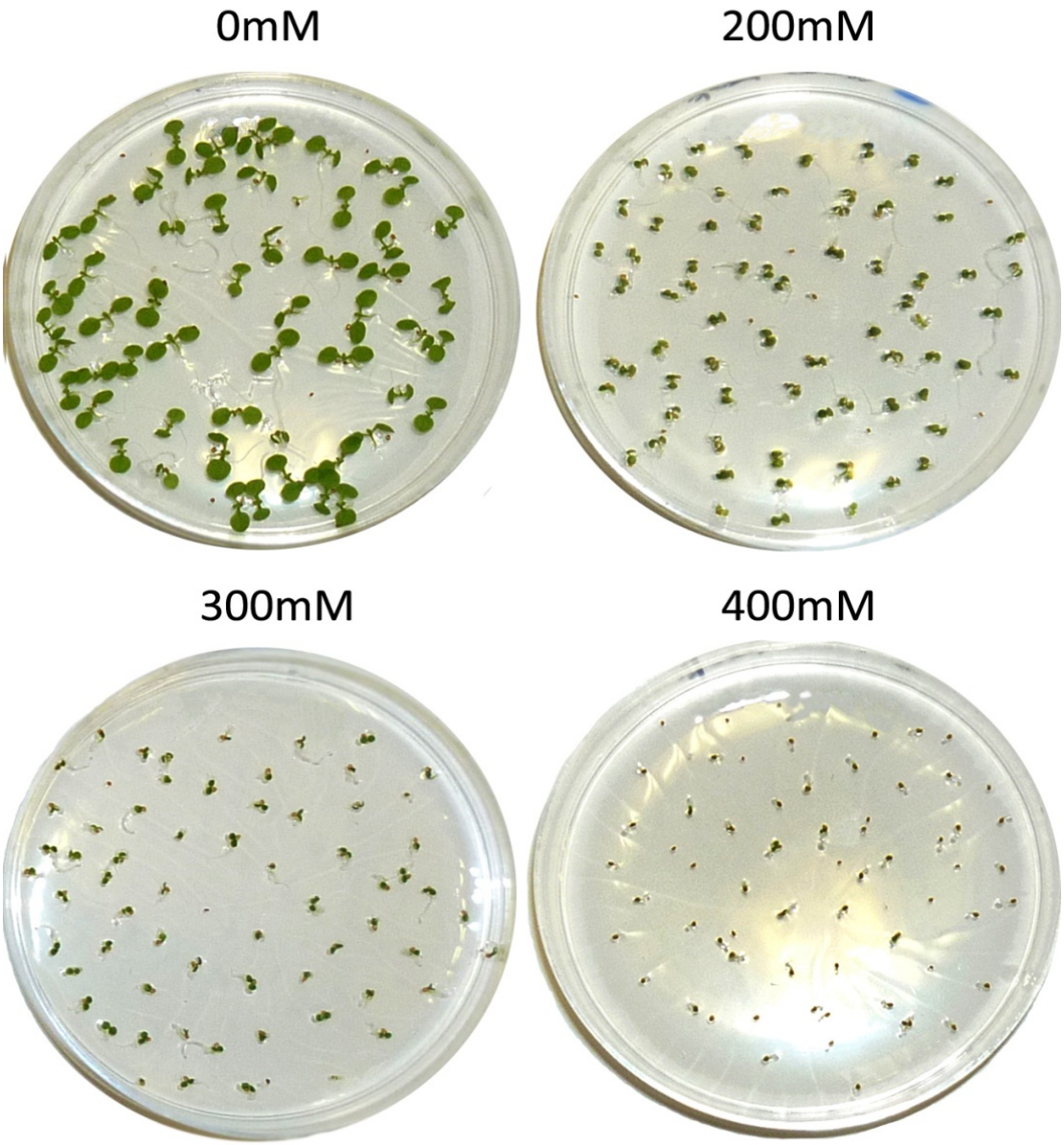


Figure 3.22. Petri dishes containing MS agar supplemented with four different concentrations of mannitol; 0mM, 200mM, 300mM, and 400mM. All seedlings were grown for seven days at 20°C in a Percival growth chamber, and were then viewed under a light microscope. Col-0 seedlings are used as a representative of what was seen for all seedlings tested.

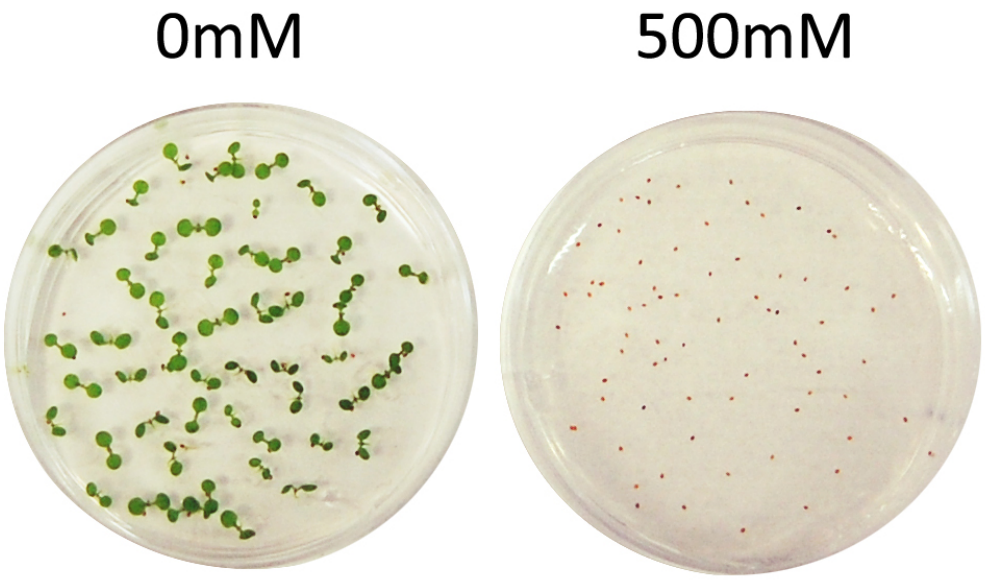


Figure 2.23. Petri dishes containing MS agar supplemented with 500mM mannitol. All seedlings were grown for seven days at 20°C in a Percival growth chamber, and were viewed under a light microscope. Col-0 seedlings are used as a representative of what was seen for all seedlings tested.

3.11.2. Effect of osmotic stress on seedlings

Col-0, *sfr4*, *sfr5* and *sfr8* were tested for osmotic tolerance when floated on various concentrations of mannitol (0mM, 330mM, 400mM and 500mM) at 20°C under the standard long day conditions for 72 hours (figure 3.24). These were conditions used in previous studies (Knight *et al.* 2008, Wathugala *et al.* 2011).

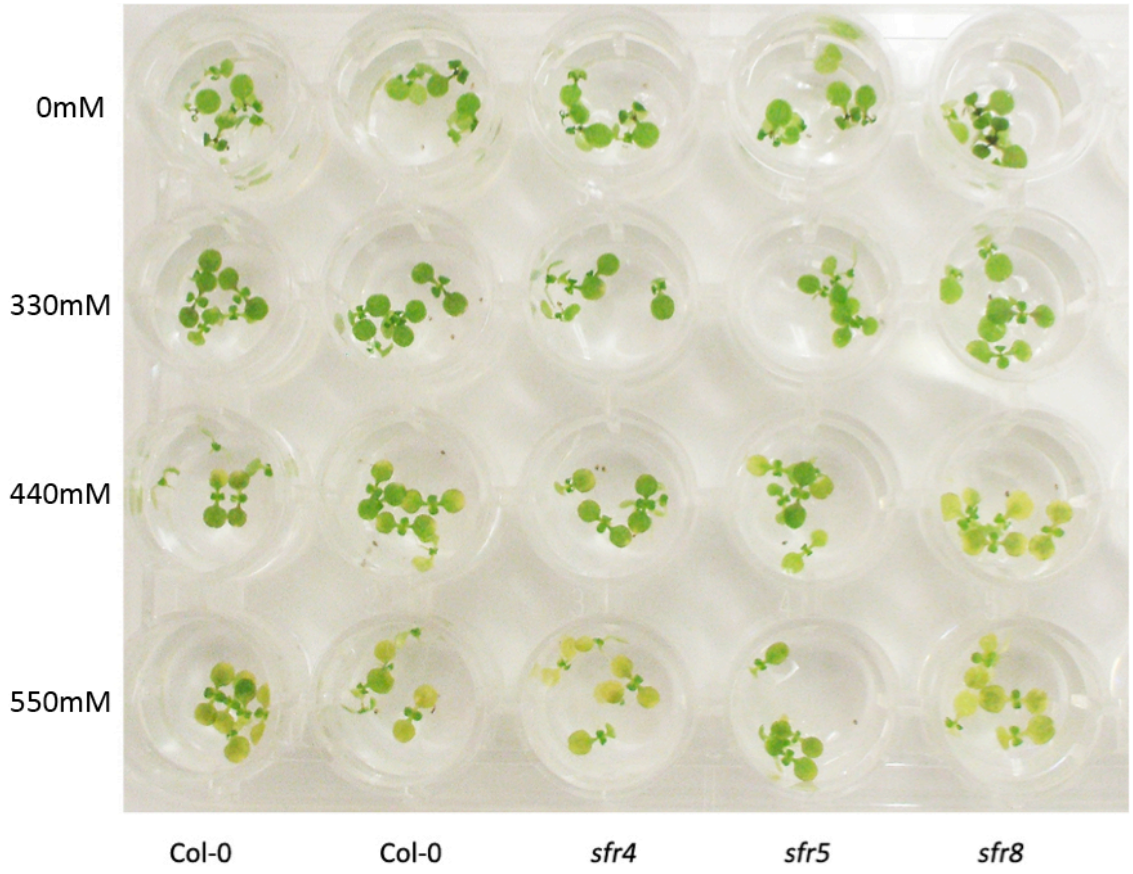


Figure 3.24. Col-0, sfr4, sfr5 and sfr8 *seedlings were floated on the indicated range of concentrations of mannitol for 72 hours and levels of chlorosis were judged against the Col-0 control.*

None of the lines showed any signs of chlorosis when in water, as was expected. *sfr4* showed a similar level of chlorosis to Col-0 with increasing concentrations of mannitol, and *sfr5* seemed to perform better than Col-0, appearing much greener even on 500mM mannitol. *sfr8* is significantly more chlorotic than the Col-0, indicating that *sfr8* may have reduced osmotic tolerance compared to the Col-0.

3.11.3. Development of a seedling freezing assay

Freezing assays on whole plants require over two months to complete; conducting freezing assays on seedlings would reduce the time required to verify if an insert line or other mutant is freezing sensitive. Traditionally, freezing assays have been conducted on adult plants (Warren *et al.* 1996) however freezing assays conducted on seedlings have been previously trialled (Xin and Browse 1998). As a result, this method was developed and compared to results seen in adult plants. If the number of seedlings that survive freezing is a) comparable to that seen in mature Col-0 plants, and b) replicable, it would suggest that this method is a reliable source of information, and could potentially be used as a trial to determine whether a full whole-plant freezing assay was worthwhile. The third criterion was to test whether the method could distinguish a known *sfr* mutant from the Col-0.

Seedlings were sown on MS agar plates and grown in a Percival growth chamber for seven days. They were then transferred to 5°C for seven days, before being transferred to -7.5°C for 24 hours. When frozen, Petri dish lids were removed as to allow cold air to circulate. The seedlings were then returned to 5℃ and then 20°C and were observed for re-growth. The plant samples this method was tested upon were Col-0 and *sfr6; sfr6* has a very severe freezing sensitive phenotype and its use in this experiment was intended to determine whether both normal and deficient seedlings respond in the same way to freezing as adult plants.

Preliminary experiments suggested that Col-0 seedlings were able to recover from freezing to a degree that may be comparable to adult plants, that cold acclimation on plates is possible, and that the assay was capable of distinguishing between a freezing sensitive mutant and Col-0 (figure 3.25). *sfr6* is naturally a much lighter colour than normal Col-0 seedlings; it is not only affected in its tolerance of freezing but in many other pathways (Knight *et al.* 2009). This difference in colouration makes no difference to the outcome of this experiment, but is just an example of what a 'healthy' *sfr6* seedling looks like. It should be noted that freezing the agar plates changed the consistency of the agar on thawing, as can be seen in the figure. However, it did not seem to have an effect on the survival of the seedlings.

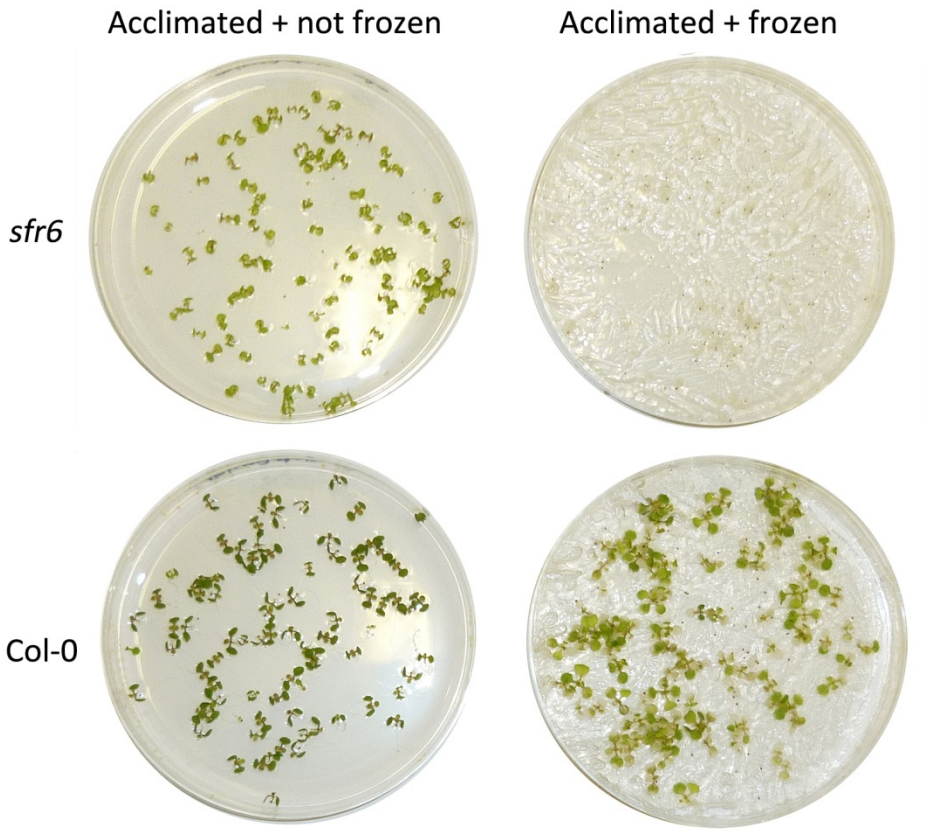


*Figure 3.25. s*fr6 *and Col-0 seedlings were grown at 20°C for seven days, acclimated at 5°C for four days, and then frozen for 24 hours at -7.5°C.After freezing they were transferred to 20°C. Photographs were taken two days after the freezing event.*

3.11.4. Dark/light regulated gene expression

It is known that *sfr4* has reduced sugar accumulation (Uemura *et al.* 2003), and it is likely that *sfr8* may have an altered perception of sugars, or an altered response to sugar. The sugar-responsive gene dark inducible 6 (*DIN6)* was used to see how these two *sfr* mutants responded to sugar. *DIN6* is inducible in the dark and repressed by light or sugars. Seven day old Col-0, *sfr4* and *sfr8* seedlings were floated on either 5ml of 0.088M sucrose or 0.096M mannitol and subjected to either six hours in the light or six hours in the dark, both at 20°C. *DIN6* gene transcript levels for each of the treatments were measured via qRT-PCR (figure 3.26).

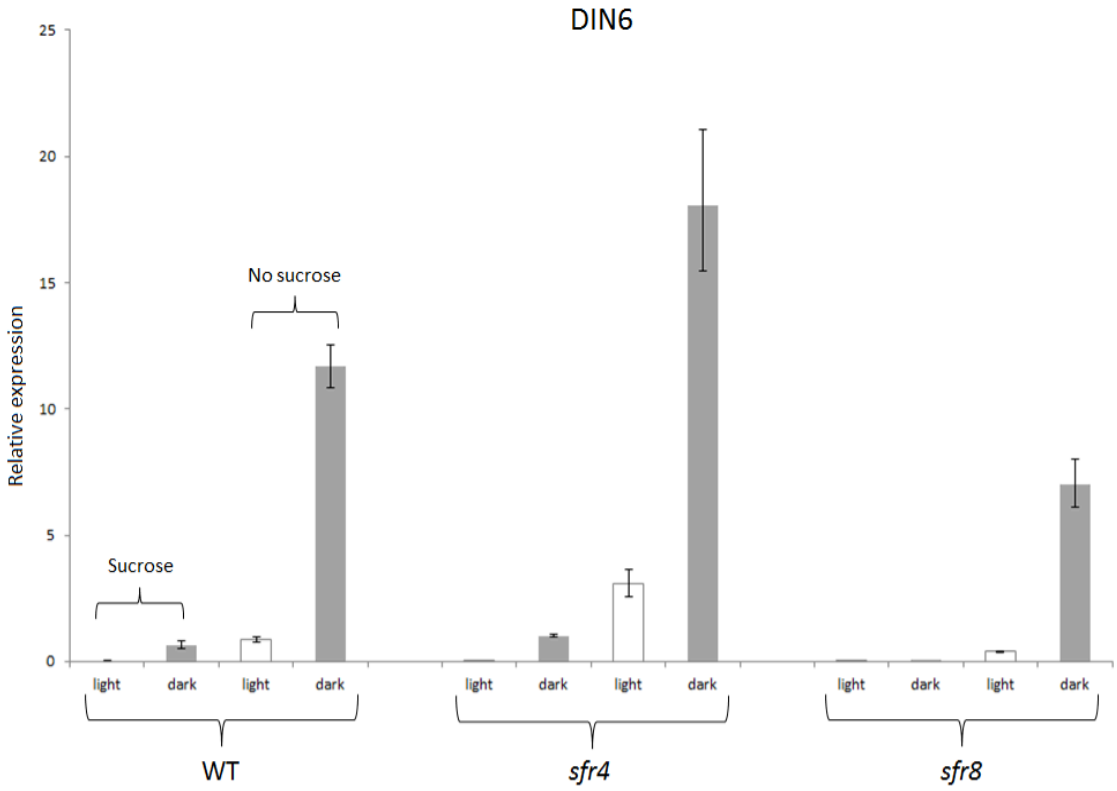


Figure 3.26. Expression levels of DIN6 in Col-0, sfr4, sfr8*, when exposed to light or darkness, and mannitol or sucrose. Samples were subjected to each of the treatments for six hours, before samples were taken.*

DIN6 is expressed in dark-treated plant tissue and is repressed by sucrose (Fujiki *et al.* 2000) as seen in the Col-0 control. Without sucrose, both *sfr4* and *sfr8* showed the expected response in both the dark (increased *DIN6* expression) and in the light (reduced *DIN6* expression). *sfr4* shows higher levels of both dark and light expression than Col-0, particularly in the absence of sucrose, where *DIN6* is evidently less repressed by sucrose than it is in Col-0. This could be as a result of the known sugar deficiency. *sfr8* shows reduced expression under all conditions compared to Col-0. This would suggest that both *sfr4* and *sfr8* show an altered sensitivity to sugar, and this is potentially worth further investigation. As this experiment has no independent biological repeats, this result would need to be replicated two further times for these trends to be considered legitimate.

3.11.5. Flowering time in *sfr8*

Throughout this investigation it was noted that *sfr8* repeatedly displayed an early flowering phenotype; however this was something that had not been reported in the literature. To test this observation, 20 individual *sfr8* and Col-0 plants were grown up alongside each other under standard long day conditions (as described in the methods) and both the number of rosette leaves present on the plant when the flower bolt was 1cm long and the date that flowering occurred were recorded for each individual. The number of leaves present on the plant is an indication of the developmental stage the plant was in when flowering occurred (Lee *et al.* 1993, Knight *et al.* 2008)

Table 3.4: Flowering time of sfr8 *compared to Col-0. The number of leaves on each plant when the flower bolt was 1cm long was measured, to the nearest leaf. Raw data was used for statistical tests. Day 0 = day seeds were transferred from the cold room to growth chamber.*

	Col-0	***sfr8***
Average no. leaves per plant when flower bolt was 1cm long (to nearest whole leaf)	17	11
Day all plants had flowered by	34	28

There is an evident difference in both the number of leaves and the day by which flowering occurs; there was a one week difference between when all *sfr8* plants and all Col-0 plants had flowered, which considering the age of these plants represent extremely different developmental stages. In figure 3.27, it is obvious that the *sfr8* are much smaller and have a lower number of leaves than the Col-0, however all *sfr8* plants had flowered by this point. Significant differences in flowering time between Col-0 and *sfr8* were seen in two independent biological repeats. For the Student's *t*-test, the P value was 2.12×10^{-20} on a sample size of n = 20, suggesting there is a statistically significant difference between the number of leaves present when the flower bolt was 1cm long.

Figure 3.27. Comparison of sfr8 *(left) and Col-0 (right) in time taken to flower. Both sets of plants were sown at the same time and were grown under the same conditions.* sfr8 *plants are much smaller than Col-0 plants, and all* sfr8 *plants have established inflorescences, whereas much fewer Col-0 plants have yet to put up a flower stalk. Photograph was taken on day 28, with day 0 being the day that seeds were transferred into the Percival growth chamber.*

3.12. Phenotypic results summary

For all three mutants, germination on mannitol did not vary from that seen in the Col-0 control. When floated on different concentrations of mannitol, there were slight differences seen between the responses of the different mutants. *sfr5* seemed less injured than the Col-0 control, whereas *sfr8* showed higher levels of damage at a lower mannitol concentration. *sfr4* showed a similar level of tolerance to Col-0.

sfr8 and *sfr4* both showed results that differed from the Col-0 control when *DIN6* gene transcript levels were measured. *sfr8* showed a potential hypersensitivity to sucrose, whereas *sfr4* showed a potential lack of sensitivity to sucrose. The difference in flowering time seen between *sfr8* and Col-0 was seen to be statistically significant, however it remains unknown if this is linked to the sensitive to freezing phenotype.

4. DISCUSSION

The original screen for sensitive to freezing (*sfr*) mutants was conducted in 1996, on a population of ethyl methanesulfonate (EMS) mutagenized Columbia 0 (Col-0) Arabidopsis (Warren *et al.* 1996). Due to the mutagenesis method, single nucleotide polymorphisms (SNPs) were the focus of this investigation. The screen was for reduced freezing tolerance post-acclimation, rather than innate freezing tolerance; all of the *sfr* mutants identified via this screen behave in the same way as Col-0 plants if frozen without an acclimation step. It is when the plants are acclimated that the difference between the freezing tolerance of the mutant and the Col-0 is seen. Arabidopsis is an ideal candidate for use in freezing tolerance screens as so much is known about how it acclimates and responds to freezing (Thomashow 2010). The use of forward genetics (the search for a genotype using a phenotype) works well in Arabidopsis, primarily due to the wealth of genetic information that is available, particularly for the Col-0 ecotype. Forward genetics studies have evidently been successful in the field of freezing tolerance; the genes responsible for the freezing tolerance deficit in *sfr2*, *sfr3* and *sfr6* have all been identified via this method (Knight *et al.* 1999, Kim *et al.* 2004, Thorlby *et al.* 2004, Amid *et al.* 2012).

The search for plants with disrupted post-acclimation freezing tolerance required a very precise freezing assay; a plant must be frozen to the point of being noticeably more damaged than the healthy Col-0 control, but the individual plant must ultimately survive and set seed to allow further investigation. This was negated in the isolation of the *sfr* mutants by the generation of sibling plants that displayed the same genotype, allowing plants to be thoroughly frozen (and thus die) without the genotype being lost (Warren *et al.*

1996). The aim of this study was to identify genes that were potentially responsible for the freezing sensitivity seen in the mutants *sfr4*, *sfr5 sfr8* and *sfr9.* Quantitative real time PCR (qRT-PCR) experiments measured expression of the C-repeat binding factors (CBFs) and the cold regulated (*COR*) genes protein kinase 2 (*KIN2)* and galactional synthase 3 (*GOLS3)* in cDNA from *sfr* seedlings subjected to cold conditions. These experiments showed that the CBF pathway is not disrupted in *sfr4*, *sfr5* or *sfr8*, indicating that all of these mutants are deficient in a different pathway leading to freezing tolerance, however a third independent biological repeat would be required to truly confirm this. There are a number of pathways that result in drought tolerance that also have cross talk with the freezing tolerance pathways, one such example is the dehydration-responsive-element-binding protein (DREB2)-mediated induction of the same *COR* genes activated by the CBFs, discussed in more detail later. Abiotic stress tolerance networks are known to converge, and it could be possible that any of the *sfr* mutants studied may have deficiencies in osmotic tolerance. As a result, this was something that was investigated.

4.1. Analysis approach

The original mapping interval of each of the *sfr* mutants (Thorlby *et al.* 1999), was further refined via personal communication with Glenn Thorlby (unpublished data). The basis of mapping relies on the fact that when a marker is close to the mutation (in terms of physical distance), the likelihood of a recombination event occurring between the two is reduced (Jones *et al.* 1997). To determine the position between the two markers and the mutation, there must be two meiotic recombination events; one between the left marker and the mutation, and one between the right marker and the mutation. It is only when this occurs that it is possible to map a mutation to a certain region.

An Illumina genome sequence was conducted for each of the *sfr* mutants. These genome sequence data were then analysed in two different ways; which method was used for which sample depended upon how successful the genome sequencing run had been, and how well the data had mapped to the reference genome. One method, the command line method, required supercomputing power, an advanced knowledge of computer programming and various specific bioinformatics software packages. The resultant dataset was a list of SNPs and their position in the genome. The second method, the Galaxy method, required very little knowledge of bioinformatics and was an almost completely automated process; the output a file that was easily viewable on the Integrative Genomics Viewer (IGV) software package, and a manual comparison of the next generation sequencing (NGS) data and the reference genome to search for SNPs was required. A low frequency of mutations was found within some areas of the genome studied, which was not expected. Often, plants mutagenised via EMS experience a large number of SNPs, however a large number of these SNPs will introduce sense mutations that have no effect on phenotype. Mutations found by both methods were verified to exist within the genomic DNA of the respective *sfr* mutant by PCR and Sanger sequencing.

A collection of mutants have been developed in which a transfer-DNA (T-DNA) insert has been introduced into the genome; the insertion is random, however most genes have several of these lines available (Alonso *et al.* 2003). For the candidate genes for each of the *sfr* mutants, further mutant alleles were obtained in the form of these T-DNA insertional mutants, if available. In these lines, the expression levels of the gene potentially knocked-out/down by the T-DNA insertion was tested. If expression levels were significantly reduced,

the new mutant allele was used in a freezing assay alongside a Col-0 control, to test whether it responded in the same manner to acclimation and freezing. It was also compared to the *sfr* mutant it potentially corresponded to.

The method used to identify the genes responsible for each of the *sfr* mutations studied in this investigation involved the use of classical mapping combined with modern techniques; next generation sequencing and SNP calling. There are advantages and disadvantages to both classical mapping and *in silico* analysis of genome sequence in a search for mutations, and also to the other methods that are available for the identification of genes from a phenotype. The advantages of this method were that a large amount of the data analyses were conducted *in silico* reducing the amount of time taken; for classical mapping to be continued, an F_2 population of Landsberg *erecta* (Ler) x Col-0 would be grown up and genotyped to refine the interval for each of the *sfr* mutants. The large number of genetic differences that exist between the Col-0 and Ler ecotypes could be used as markers to identify a region in which each of the *sfr* mutations is found.

The downside of this mapping technique is that it can take an extremely long time. It can take many years to get from the EMS mutant to the gene in which the mutation occurs, even when working with the short generation time of Arabidopsis, which speeds up the genotyping of F_1 and F_2 generations. This method would not be possible for *sfr9* as the only available resource for this mutant is genomic DNA. It has also been previously shown that in areas of low recombination (as were seen with *sfr6*), classical mapping can only go so far as to determine an interval in which a mutant exists and fine mapping is virtually impossible (Knight *et al.* 2009).

Another potential technique, which blends classical mapping with the use of next generation sequencing, is SHOREmapping. Like traditional mapping, it requires the original mutant to be crossed into another Arabidopsis accession (i.e. Ler), an F_2 population must be obtained, and the genomic DNA of 500 pooled F_2 plants that segregate for freezing sensitivity are subjected to bulk sequencing via Illumina next generation sequencing (Schneeberger *et al.* 2009). The read data is compared with the reference sequence, and the data are virtually mapped using the known markers existing between Col-0 and Ler. Using these data, the SHOREmap software finds an interval in which only Col-0 DNA is present, meaning that the mutation causing the phenotype will be found within this interval. SHOREmapping software is used to refine this interval, and the final result is an extremely small number of SNPs that may be responsible for the mutant phenotype (Schneeberger *et al.* 2009).

While SHOREmapping does still require the basic level of mapping, (mutant plants must be grown and crossed with Ler plants), when an F_2 population is achieved the process transitions into mapping-by-sequencing. However, conducting freezing tests on 2000 individual F_2 plants in order to find the one quarter that were freezing sensitive would be no trivial matter. Much like continuing with classical mapping, this technique would not be suitable for use with *sfr9.*

Were two alleles present for each of the *sfr* mutants involved in this study, next generation sequencing runs could be conducted for the two separate alleles, and the gene which displayed two different mutations would be pulled out as that responsible for the freezing

sensitivity. This is not something that could be conducted for the *sfr* mutants, as all only have one available allele.

4.1.1. Sequencing and bioinformatics

Next generation sequencing data were obtained, involving the preparation of a genomic DNA library, which was then sequenced via the Illumina method. An entire genome sequence was produced for each of *sfr4*, *sfr5*, *sfr8* and *sfr9*. Due to the EMS mutagenesis treatment each of the *sfr* mutants was subjected to, it was known that a higher number of SNPs would be present within the mapping interval for each *sfr* mutant than would be causing the sensitive to freezing phenotype. Likewise, there would also be benign, naturally accumulated SNPs that were found in all individuals of the specific Col-0 population that was used to generate the *sfr* mutants. Only one of these SNPs would be responsible for the freezing sensitivity, however there would be a great number of silent mutations introduced into the genome. There were a large number of false positives in this investigation, with very little way of determining what was a 'legitimate' candidate and what was not without using PCR and Sanger sequencing. A number of criteria (methods section 2.11.4) were applied to the two mapping methods in an attempt to remove as many of these false positives *in silico*. Additionally, a SNP that occurred in more than one of the *sfr* mutants could be discounted as it was extremely likely to be a population-specific SNP.

The sequence data received were of various different qualities, and it became evident that Illimuna sequencing cannot always provide adequate sequencing depth required for the Galaxy method. Regions near centromeres, tandem repeats, and sequences that occur more than once in different parts of the genome can all reduce the efficiency of the alignment of

sequence data to the reference genome (Treangen and Salzberg 2011). It could be that these areas occur over region to which a particular *sfr* mutation has been mapped. In this instance other techniques may have to be adopted, such as sequence capture, where oligos are designed to a certain region rather than sequencing the whole genome, and this region is enriched and given a deeper level of coverage (Hoppman-Chaney *et al.* 2010).

4.1.2. Methods used for bioinformatics analysis

The Galaxy method is widely used and accepted as a feasible way of pulling SNPs out of the Illumina data (Hillman-Jackson *et al.* 2002). The method is time-consuming, and can be liable to human error in the mis-recording of SNPs, however it does give a visual representation of the genome meaning it is very obvious if a region has not mapped well to the reference genome. The original dataset received for *sfr4* was a good example of this advantage. With this method it is possible to tailor the mapping software Bowtie to the requirements of the user, and this is something that may yield better mapping results if repeated multiple times with slight changes to the settings (Langmead *et al.* 2009).

Human error is a negligible factor using the command line method, however the method employed was not dedicated to this specific type of SNP-calling; which entailed locating purely homozygous SNPs. When the *sfr5* data were analysed, there were seven (table 3.1) heterozygous SNPs within genes. All were proven to not exist within the genomic DNA of *sfr5* in the DNA, bar one which was not tested on the grounds of how low the likelihood of it existing was**.** Likewise, the criteria of the command line method are biased towards high levels of genome coverage; as part of the criteria used in the command line method, areas of the genome must have 10x coverage for a SNP to be considered legitimate. This resulted

in SNPs that were found via the Galaxy method and were proven to exist within the DNA (e.g., the *sfr8* SNP in murus 1 [*MUR1*]) failing to be identified via the command-line method. Just because an area of genome displays low coverage, this does not mean that any mutations found within these regions are not legitimate; all of these must then be retested within the genomic DNA to determine whether they are legitimate.

The comparison of the same data using both methods is the most interesting with regards to this; it was of interest to see if both methods yielded the same SNPs as likely candidates. The overall number of SNPs the command line picked up on was lower than those found via the Galaxy method; for *sfr8*, two of the candidates genes that were investigated were found via the command line method, however *MUR1* was not. The region of the genome in which *MUR1* exists did not display as much sequence depth as other regions, and as a result probably did not meet the criterion for genome coverage that was present in the command line method. As a result, a suggestion in modifying the command line method for this region of the genome would be to lower the stringency, and accept regions of lower coverage. Likewise, the mutation found in the *sfr9* genome sequence was not identified by the command line method; in fact, the command line method did not detect any potential candidates for *sfr9*. This was also the case for the re-sequenced *sfr4* data.

While the command line method has produced fewer SNPs for the two Illumina datasets where the data were of sufficient quality to be analysed by both methods, it should not be forgotten that for *sfr5*, the command line method was the only method that yielded SNPs. The data for *sfr5* did not map well with Galaxy, and it was not possible to pull SNPs out of the data it provided. If possible both SNP identification methods should be used, as the

command line method is potentially better at mapping the raw NGS data to the genome. With reduced stringency levels, it could be even more successful.

Providing the raw data are of high enough quality, the Galaxy and Bowtie online platform allow a user with very little experience of bioinformatics to successfully identify legitimate candidates genes for mutations caused by SNPs. Having proven that the Galaxy method is able to successfully identify SNPs is extremely useful, as next generation sequencing is becoming an extremely useful tool, and the ability for an end-user to do their own data analysis is crucial.

4.1.3. T-DNA Insert lines

SALK and GABI-Kat T-DNA lines are a cheap and relatively efficient method of knocking out or down the potential candidates from the SNPs identified from next generation sequencing data (Alonso *et al.* 2003). This project depended heavily upon the use of SALK and GABI-Kat T-DNA insertional lines, and throughout the process it was discovered that there were various problems that could occur and hence hinder progress. Even after the process of genotyping an insert line (which in itself can be time consuming, and isolating a homozygote can take several months) there is no guarantee that the insertion will cause a change in expression of the transcript. This was a problem with some of the lines that were available. Insertions were in extremely poor positions in the gene, including intragenic regions, too far into the untranslated regions, or in introns. Throughout this investigation there were several cases when a SALK line failed to reduce the expression of the gene, thus rendering it useless. Conversely, it was sometimes seen that the T-DNA insert could bring about increased levels of expression for a given gene; this is a known problem due to the CamV 35s promoter used

to insert the T-DNA (Ülker *et al.* 2008). Another problem was that for some genes, there was no available T-DNA insert line, as was the case for the *sfr5* candidate, At1g15410.

Even when insert lines were available, several lines displayed weak germination and survival rates, meaning that only one qRT-PCR experiment could be conducted when testing the effect of the T-DNA insert on the expression of the gene. Due to time limitations it was not possible to allow these plants to self-pollinate and grow up enough progeny to allow repeats of the qRT-PCR tests, however due to the nature of the knockdown mutant, i.e. the gene has been inactivated by an insertion into the gene which should be present regardless of what abiotic conditions the plant is subjected to, I believe these result are sufficient to make an informed judgement on the efficiency of the inactivation of the gene.

It may not always be preferable to completely eradicate a gene transcript; difficulties were encountered when genotyping the insertional mutants for the *sfr8* candidate, *MUR1*. No homozygote individuals were successfully obtained. The known *mur-1* and *mur-2* mutants have extremely low levels of L-fucose (Zablackis *et al.* 1996), but production is not completely knocked out; it is possible that a complete knockout (as may be caused by a T-DNA insert line) may be lethal.

If only one insertional mutant is available and it successfully knocks down the gene, this is not sufficient to confirm linkage and it is preferable if the mutant can be complimented with a wild type sequence. As a result, to be completely confident in the result of an insertional mutant, it may be preferable to supplement the results using an RNAi knockdown. The results of RNAi are often not as extreme as T-DNA insertional lines; the gene in question is

often knocked-down rather than out, however this can be advantageous when a complete knockout is lethal, and is a valuable addition if no T-DNA insert lines are available. RNAi is, however, a more labour intensive method. A fragment to be focussed on must be chosen, cloned into the appropriate vector and infiltrated into the plant tissue. Certain fragments do not successfully transfer into vectors on the first attempt, which can result in the process becoming time consuming. This method is not particularly feasible for a large number of candidates, and it was not possible to create RNAi clones and successfully transform wild type plants on the timescale of this investigation.

4.1.4. Plant freezing assays

While extremely useful, the standard freezing assay is slow and laborious, and can sometimes yield unexpected results, including inconsistencies between repeats. There are an extremely large number of variables that can affect the results of a freezing assay and minimising variability is the key to producing replicable results. Conventional freezing assays on adult plants take a number of months to complete, and as a result finding a quick and easy of way of verifying the freezing sensitivity of plants while at the seedling stage would have been very beneficial. An assay that did just this was developed, however this occurred at the very end of the period of study and as a result was not used. It may, however, prove useful in future investigations of this sort.

One particular problem with freezing assays is choosing a freezing temperature that will give reproducible results, i.e. a temperature that will always result in the death of freezing sensitive plants. Likewise, the position and number of individual plants within the freezing chamber can have an extreme effect on the survival of plants. *sfr5* is known to display a less

severe phenotype than other mutants tested (McKown *et al.* 1996), which makes freezing assays more challenging, as it was seen on one occasion that *sfr5* survived freezing conditions that had been previously shown to be lethal. This occurred when the growth chamber contained a larger number of plants than usual, suggesting that overcrowding resulted in raised the temperature around the plants, or protected plants from ice formation. In initial investigations, plants were transferred from a 5°C growth chamber to a freezing chamber, pre-cooled to -7.5°C. This is an extremely unnatural situation, and it is known that Arabidopsis plants growth differs between environmental growth chambers and an outdoor environment (Mishra *et al.* 2012). The sudden shock of such a substantial drop could potentially cause more damage than a gradual cooling to this temperature; this is something that has been shown in other species (Finkle *et al.* 1974). Transferring the plant from the 5°C growth chamber to the -7.5°C freezing chamber when the temperature is decreasing could be an option.

Edge effects and differences in air flow within the freezing chamber were a concern, and attempts were made to control these to some degree by randomising plants of different lines within the tray. Placing the peat plugs on trays that allow air curculation may be an alternative method to prevent insulation problems.

By the time adult rosette plants are suitable for freezing tests, it is often the case that they have become root-bound by their peat plugs, and that roots have managed to penetrate the outer casing of the plug, leaving them exposed when subjected to freezing temperatures. This potential root exposure is not a situation that the plants would experience in nature, and as a result the damage that occurs to the roots may have a significant effect upon their

recovery. Somehow, protecting the roots of adult plants while they are frozen may produce more accurate, reproducible results.

Throughout this investigation it was discovered that plants of different ages showed that their responses to freezing might differ. Plants that were four weeks old when they were put into acclimation were less likely to recover from a freezing event than plants that were five weeks old, suggesting that further investigations into optimal plant age are required.

The results of a freezing assay could be supported by electrolyte leakage assays (Jaglo-Ottosen *et al.* 1998). The one advantage of electrolyte leakage assay is that leaves are tested over a range of freezing temperatures, so it is not crucial to find an optimum freezing assay temperature. However, this is not something that could have been conducted on all of the candidate mutants due to live plants being unavailable for *sfr9*.

4.2. Results from each of the mutants

4.2.1. *sfr9*

Due to the fact that the *sfr9* mutant no longer germinates, nothing can be done in the way of phenotypic investigations. Genomic DNA used for the genome sequence was extracted from seeds. The one mutation found for this mutant (via the Galaxy method) is between two genes and is potentially within the promoter region of glucosinolate transporter 2 (*GTR2)*, however, it may be too far away from the closest gene to have any effect upon its expression. This area of the genome was checked for ESTs and none were found, and it was verified that throughout the mapping interval for this mutant, the genome sequencing coverage was complete and without gaps. As the mutation causing the *sfr9* phenotype must

be within this interval, there is very little that could be done with this mutant. One suggestion would be to re-sequence this region using the sequence capture method discussed previously, focussing specifically upon the mapping interval.

4.2.2. *sfr4*

The deficit in freezing tolerance in *sfr4* has been shown to be due to a significantly reduced accumulation of sucrose during cold acclimation when compared to Col-0 plants (Uemura *et al.* 2003). When an *sfr4* plant has been frozen without acclimation, levels of sucrose are not thought to differ from those seen in Col-0. *sfr4* has also been shown to not accumulate anthocyanin to the same levels as the Col-0 in response to cold (McKown *et al.* 1996, Uemura *et al.* 2003). Anthocyanins, which are antioxidants that protect plants from damage by reactive oxygen species (Nagata *et al.* 2003), have been shown to accumulate in response to increased sucrose levels. Therefore, this lack of anthocyanin in *sfr4* could be the product of the sucrose deficiency (Teng *et al.* 2005). The dark/sucrose gene expression experiment (3.26) suggests that either the perception of sucrose, or the cellular levels of sucrose have been altered in *sfr4,* which is consistent with previous findings.

sfr4 showed no unusual germination response compared to Col-0 when grown on mannitol-supplemented media, nor when seedlings were floated on various concentrations of mannitol. These results indicate that the freezing-sensitive phenotype of *sfr4* is not accompanied by an osmotic sensitivity (Warren 1996). The use of compatible solutes to prevent water loss from cells occurs in both droughted conditions and when freezing of plant tissue occurs. As a result, this would suggest that when plants have been grown under

ambient temperature conditions, the freezing and osmotic tolerance of *sfr4* should be equivalent to that of Col-0.

It has been previously proven in protoplasts that *sfr4* has a deficit in sucrose, and that supplementing the mutant with sucrose results in the restoration of freezing tolerance (McKown *et al.* 1996). Using protoplasts is not always an accurate representation of the response on a whole plant scale, and as a result the intention was to use the Petri dish freezing assay, with sucrose-supplemented media, to see if *sfr4* seedlings showed the same freezing deficit when sucrose was readily available. However, this was not performed due to the late development of the assay, and is a suggestion for further work.

Although *sfr4* is the most well studied of the four *sfr* mutants involved in this investigation, and as a result was expected to be the easiest to identify, no candidate genes were isolated. The initial sequencing run provided data that were of low coverage to the point of being unusable, however, even when the Illumina sequencing was repeated, the command line method was unable to find any candidate genes from the new data. Due to the late availability of the new data, they were not analysed via the Galaxy method. Even when the mapping interval for *sfr4* was extended, as the original interval was based on only one recombinant for one of the flanking markers, no further candidates were found. It is possible that, though the overall genome sequence coverage was high, regions within the mapping interval that contained *sfr4* were areas that were under-represented in the sequencing, and as a result the SNP causing the sfr4 mutation could simply not have sequenced, or had so few reads that it was not able to be taken as a confident SNP.

Despite the lack of candidates, when every gene in the *sfr4* interval was surveyed, none seemed to have any obvious links to sucrose, even though the mutation is present within this region. This suggests that the pathway that is disrupted in *sfr4* may be more complex than simply governing the accumulation of sucrose.

4.2.3. *sfr5*

sfr5 has no reported phenotype other than its freezing deficiency, and of the *sfr* mutants it shows the weakest deficiency in freezing tolerance; the sensitivity phenotype is sometimes variable (McKown *et al.* 1996). When originally isolated, two alleles for *sfr5* were identified and were found to be co-dominant (Warren *et al.* 1996); one allele *sfr5-1* is the allele focused upon in this experiment, and it is homozygous. Unfortunately *sfr5-2* seeds were no longer available at the start of this investigation; if both alleles had undergone genome sequencing only gene(s) which contained SNPs in both genomes could have been focussed upon. This would have greatly limited the number of candidate SNPs that may have been responsible for the phenotype.

At the seedling emergence stage *sfr5* appeared to be affected to the same extent as Col-0 when grown on mannitol-supplemented media, but no more. When seven days old, seedlings responded better than Col-0 when floated in mannitol, and suffered less chlorosis. *sfr5* appears more tolerant of osmotic stress than Col-0. This was not the result expected, and could potentially suggest a disrupted osmolyte pathway. The freezing sensitive phenotype seen in *sfr5* was the weakest of the mutants tested, and under certain conditions could be seen to disappear; this is discussed in section 4.1.4.

Due to the poor sequence data quality for this mutant, these data were only analysed using the command line method. The two potential genes found for this mutation were *At1g15410* and *AVP1,* a H^+ translocating inorganic pyrophosphotase. Through the use of one confirmed homozygous GABI-Kat line, it was found that an *avp1* mutant responded to freezing stress in a similar way to Col-0 plants, and as a result it would appear that a mutation in *AVP1* was not responsible for the freezing sensitivity. Due to the severity of the homozygous *avp1-1* mutation, this is not wholly unexpected; *avp1-1* is unable to flower and hence cannot set seed (Li *et al.* 2005), and this is something that has never been seen with *sfr5*. It is possible that while AVP1 expression had been knocked out in the GABI-Kat mutant, there was enough protein remaining for it to be functional, as none of the phenotypes associated with *avp1-1* were seen with the T-DNA *avp1* mutant. As a result this would suggest that further work be carried out upon this candidate.

The remaining candidate for *sfr5* was not investigated due to unavailability of SALK or GABI-Kat insert lines. Of those data supplied via the command-line method, At1g15410 is currently the only other prospective candidate for this mutant; all weaker mutations were proven not to exist in the DNA of the *sfr5* plant. The next course of action, had time permitted, would be to make an RNAi knock down of this gene. At1g15410 is a hypothetical protein that has been suggested to be located in the chloroplasts, and has been proposed to be part of an aspartate-glutamate racemase family. Not much is known about the function of this gene, however it has been found that aspartate racemases and glutamate racemases are enzymes which have been found to show high levels of similarity to each other in bacteria, and that they do not require a co-factor to function. (Gallo and Knowles 1993) Glutamate racemase catalyses the conversion of D-glutamate to L-glutamate, and vice-versa

(Gallo and Knowles 1993), and they do not need a co-factor to function. In bacteria, glutamate racemases are essential for cell biosynthesis, and D-glutamate is necessary for the synthesis of peptidoglycan found in the cell wall (Fisch 2009). Likewise, aspartate racemases convert D-aspartate to L-aspartate, and also does not require a co-enzyme (Yamauchi *et al.* 1992). It is possible that the involvement of these proteins in bacterial cell walls could infer an involvement of At1g15410 in the plant cell wall.

4.2.4. *sfr8*

There was no prior knowledge as to the cause of the freezing tolerance deficit in *sfr8*, other than that the mutation was recessive and caused a loss-of-function. The CBF pathway was shown to be functioning as normal (figure 3.1), and the osmotic tolerance assay for those both grown on plates and floated on solution showed similar results to the Col-0 control (figure 3.24).

During the investigation it was noted that *sfr8* flowered much earlier than Col-0; in a sample size of twenty, *sfr8* plants flowered an average of seven days earlier than Col-0 under long day conditions. This phenotype was seen under ambient conditions, with no potential causes of stress. Flowering and freezing sensitivity phenotypes often appear in the same mutant*; sfr6* is known to flower later than Col-0, due to reduced expression of genes involved in the photoperiodic flowering pathway (Knight *et al.* 2008). It is likely that there are links between the flowering time pathway and freezing tolerance. One gene, Long Vegetative Phase 1 (*LOV1),* links freezing tolerance to flowering time and, when mutated, results in delayed flowering under long day conditions (Yoo *et al.* 2007). LOV1 is a plant-

specific NAC-domain transcription factor, which has been shown to negatively regulate Constans (*CO*) and Flowering Locus T (*FT*), two integral components of the flowering pathway (Turck *et al.* 2008). Alongside this regulation of *CO*, it has also been shown to be involved in the cold response. The *lov1-4* null mutant was shown to display reduced freezing tolerance, whereas overexpression of LOV1 caused increased expression of the genes Cold-Regulated 15a (*COR15A)* and *KIN1*. In the *lov1-4* mutant, *COR15A* was down-regulated, however *KIN1* was unaffected, suggesting LOV1 is not required for *KIN1* expression. It was theorised that LOV1 may bind to the CRT/DRE of *COR* genes. Another late flowering mutant is High Expression of Osmotically Responsive Genes 9 (*HOS9). HOS9* encodes a homeodomain transcription factor, and it is suggested to be responsible for constitutive freezing tolerance, as *hos1* mutants are more sensitive to freezing before and after acclimation (Zhu *et al.* 2004).

Of the *sfr8* candidate genes, At3g50910 was proven to not be the cause of the *sfr8* mutation. Two confirmed homozygous SALK lines were tested for expression levels and were proven to successfully knock out the expression of the Col-0 gene. These SALK lines were tested via a freezing test, that even when expression of the gene containing the T-DNA was significantly reduced, the T-DNA mutant plants responded to freezing in a similar way to Col-0. The gene UDP-Glucose Pyrophosphorylase 3 (*UGP3)* was not rejected as a candidate, however due to germination problems with the T-DNA insert line used, its sensitivity to freezing when the gene was knocked out was not freezing tested.

The remaining *sfr8* candidate of those tested, *MUR1*, encodes the enzyme GDP-D-mannose-4,6-dehydratase, which is responsible for catalysing the first step in the de novo synthesis of

GDP-L-fucose, a precursor of L-fucose (Bonin *et al.* 1997). L-fucose is present in glycoproteins and polysaccharides found in the plant cell wall. Previously generated *MUR1* EMS mutations, *mur1-1, mur1-2* and m*ur1-3* were used in this investigation (Reiter *et al.* 1993). *mur1-1 and mur1-2* are extremely similar; both show less than 2% of the L-fucose seen in Col-0 plants in aerial tissues, but only a reduction to 40% of Col-0 levels in roots (Bonin *et al.* 1997). Both are slightly dwarfed, *mur1-2* slightly more so than *mur1-1*. This dwarfism compared to the Col-0 control can be seen in the freezing assay conducted upon them (fig 3.17). *mur1-3* is less badly affected, with ~33% L-fucose in aerial tissues (Reiter *et al.* 1997).

In *mur-1* and *mur1-2*, the 98% reduction in L-fucose levels has been shown to be non-lethal, as L-fucose is substituted with L-galactose in the mutant plants (Zablackis *et al.* 1996). While this substitution may function under normal conditions, under freezing conditions the structural differences between the two compounds may cause the cell wall to weaken and fail. *mur1-3* reacts to freezing in a similar way to Col-0, suggesting that if the plant is able to synthesise some L-fucose but not to the normal level seen in Col-0 plants, the cell wall is still functional under normal conditions.

The missense mutation introduced into *MUR1* in *sfr8* is G to A, which is characteristic of EMS mutations. The SNP corresponds to an already existing *mur1* mutant, *mur1-4*, which has been previously described as having an extremely low L-fucose content compared to the Col-0 control, proving that this exact amino acid substitution has a detrimental effect on the function of this protein. The area of the gene in which the mutation occurs is extremely

conserved, and as a result must be important to the correct functioning of MUR1 (Bonin *et al.* 1997).

There are a number of experiments that could be conducted to either confirm or remove *MUR1* as a candidate for *sfr8*. The most obvious of these would be to supplement the growth media of *sfr8* seedlings throughout their growth, then subsequently subject them to a freezing assay. If this restored freezing tolerance in the adult plants, it would implicates that *MUR1* is *sfr8*. There are also a number of phenotypes that are associated with the *mur1* mutants that have not yet been verified to exist within *sfr8*. The freezing sensitivity has been confirmed, but would need to be repeated for true reliability; furthermore, electrolyte leakage tests would be a useful addition in comparing the two. The flowering phenotype that is seen in *sfr8* could be measured in *mur1* mutants, however this phenotype has not previously been reported for *mur1* mutants, and as a result could be due to a SNP induced by the EMS that is independent of the gene causing the freezing sensitivity.

Two strong alleles of *mur1; mur1-1* and *mur1-2* have been tested and, as no homozygotes were successfully obtained for the T-DNA insertional lines, possibly due to the lethal nature of a complete *MUR1* knockout (Bonin *et al.* 1997), an RNAi knockdown may be more appropriate. Due to the deficit of L-fucose caused by the *mur1* mutation, growing on media containing fucose restores the phenotype of the *mur1* mutants (Freshour *et al.* 2003), and this is something that could be attempted with *sfr8* and could strengthen the case of this candidate being responsible for the freezing tolerance deficit in *sfr8*.

mur1-1 and *mur1-2* mutants have slightly weaker inflorescences and stems than normal plants; this was not something that was confirmed in *sfr8* due to the fact that when plants

began to flower, they were fitted with the Aracon system to prevent cross-contamination of seed. There is also a greater internode length in *mur1* plants compared to Col-0. This is something that could warrant further investigation. Another observation previously made about *mur1* mutants, was that root cell elongation is somewhat impaired, even though the fucose deficiency is not as severe in the roots as the shoots. This is something that could be measured in *sfr8* via microscopy (Van Hengel and Roberts 2002). Conducting the petri dish freezing assay on both *mur1-4* and *sfr8* but with L-fucose-supplemented media is an option for further work; if the dwarf phenotype and cell wall sensitivity were removed, this would give evidence that *MUR1* is *sfr8.*

The results for *DIN6* expression suggest that *sfr8* is hypersensitive to sugars (figure 3.26), which could correspond with the hypersensitivity sucrose signals that have previously been recorded in *mur1* (Li *et al.* 2007), providing more evidence that *sfr8* may be caused by a *mur1* mutation. When sucrose is added, the gene expression is somewhat more repressed in *sfr8* than in Col-0. Similarly, when sugar is not added, *sfr8* acts in the way Col-0 acts when sugar has been added. It is a possibility that *sfr8* is responding to the small amount of sugar that will already be present in the plant, acting in the way that a supplemented Col-0 plant would (Li *et al.* 2007).

4.3. Final summary

A number of gene candidates were identified for each of the *SFR* genes except for *sfr4*, and were verified to exist within the DNA of the *sfr* mutant to which they corresponded. Throughout the course of this investigation, more information has been gathered about the responses of several of the *sfr* mutants to different abiotic factors. For one, it has been confirmed that none of the three mutations tested affect the CBF pathway. While it could be argued that a gene downstream of the CBFs could be knocked-out in the *sfr* mutants , it is unlikely that one defective COR gene would result in the complete loss of freezing tolerance seen in the *sfr* mutants. The CBFs are the best-known factor involved in freezing tolerance, however they may only control 12% of the cold induced transcriptome (Fowler and Thomashow 2002). It is evident that a functioning CBF pathway alone cannot confer freezing tolerance, and as a result there must be a number of other factors involved.

In terms of candidate genes for each of the *sfr* lines, both *sfr4* and *sfr9* have resulted in no candidates. The lack of candidates for *sfr4* may be due to the higher stringency levels of the command line method, and may be able to produce SNPs if the stringency is lowered, however, it is unknown how a candidate will be found for *sfr9,* as there appears to be no apparent reason as to why one has not been discovered. *sfr5* is still potentially left with two candidates that required further investigation. However, due to the fact that the *sfr5* data could not be analysed with both methods, it is possible that there are SNPs that the Galaxy method would have picked up on had the data quality been better.

sfr8 was the most successful of all the *sfr* mutants studied, with three candidates still remaining. The freezing sensitivity of plants lacking UGP3 remains unknown, and as a result it cannot be ruled out. There is also At3g56590, which was identified too late to be of any

use in this investigation which warrants further work. However, at this point *MUR1* appears to be the strongest candidate to be causing the freezing sensitivity, with a known knockout proven to exist at the exact same base change as that seen in *sfr8*; further tests on the known *mur1* EMS mutants with regard to their freezing sensitivity is strongly recommended.

5. APPENDICES

5.1. Primer sequences

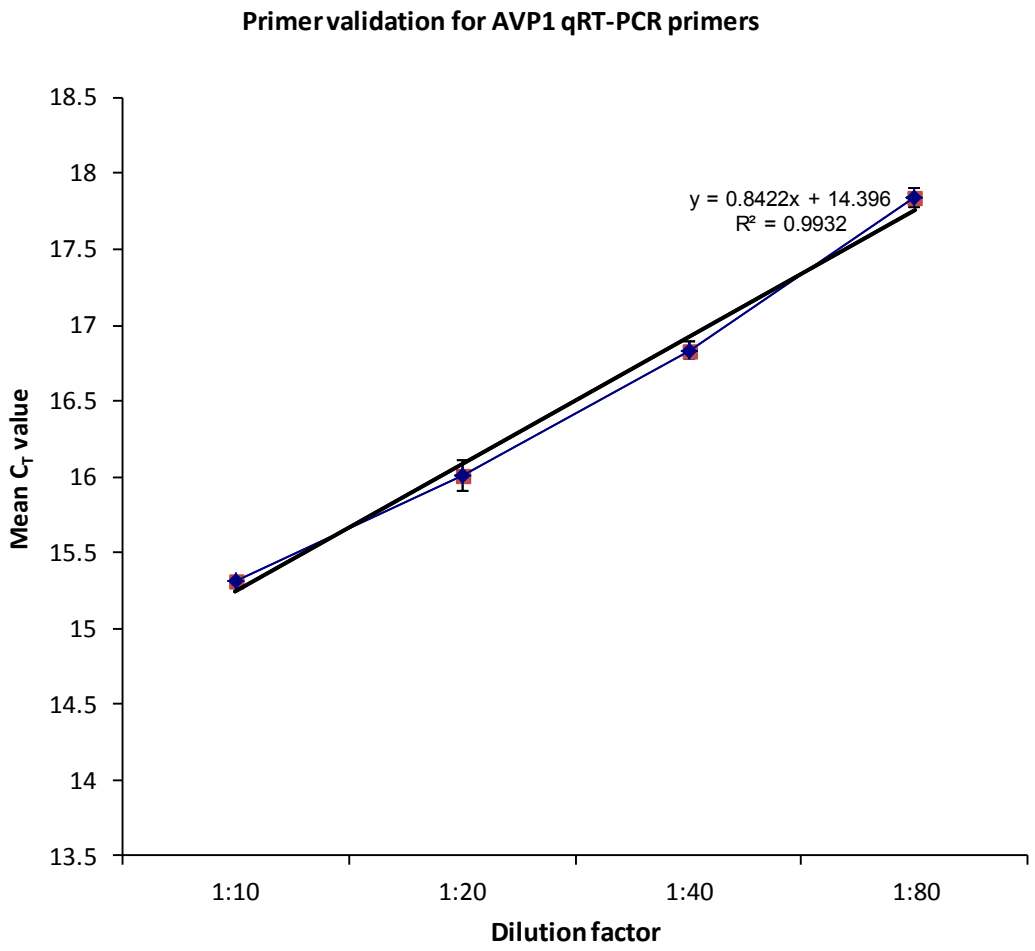
Sequence	Function
Primers looking for *sfr8* mutations	
CCATGCCTACTTTTTCTGCT	Forward primer to verify that there was a mutation in the gene At3g50910
AAGCATGACAAGGCGCTACT	Reverse primer to verify that there was a mutation in the gene At3g50910
TTCTCGGAAAAGGCTACGAA	Forward primer to verify presence of a SNP mutation in the gene At3g51160
TTGCTGCTTAGCATCCATGT	Reverse primer to verify presence of a SNP mutation in the gene At3g51160
TGGAAAAAGCTATTGCTGCTG	Forward primer to verify presence of a SNP mutation in the gene At3g51780
TCTCCAAAACTCGTAGCCATAA	Reverse primer to verify presence of a SNP mutation in the gene At3g51780
CTGGTGTTGATTGCAAATGG	Forward primer to verify presence of a SNP mutation in the gene At3g56040
TGATTTTGCACTCGAAGCTG	Reverse primer to verify presence of a SNP mutation in the gene At3g56040
TTGAAACCTGTGGAGTTCAGG	Forward primer to verify presence of a SNP mutation in the gene At3g56590
TTCCCAAACAAGGATTCAG	Reverse primer to verify presence of a SNP mutation in the gene At3g56590

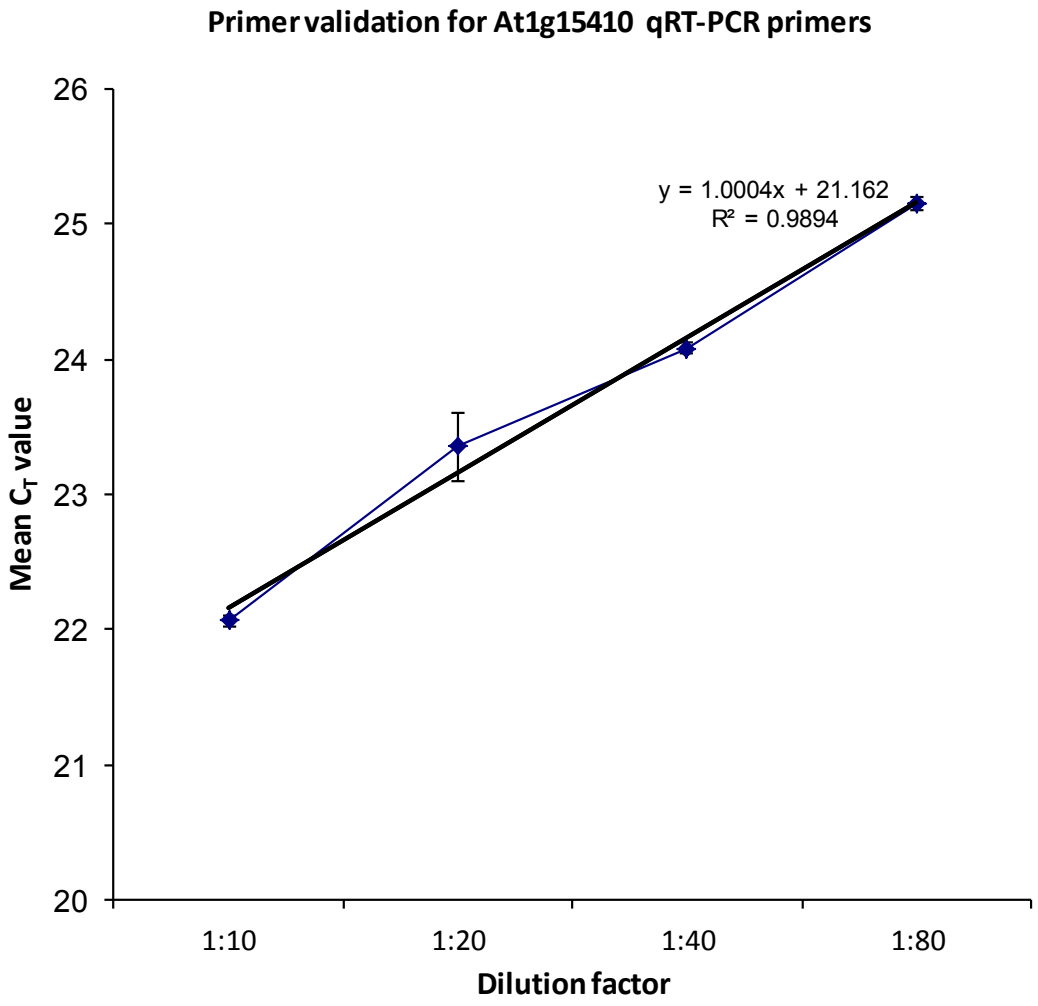
Sequence	Function
Primers looking for *sfr5* mutations	
ACCGTCTTCAGTCCTCTTGC	Forward primer to verify presence of a SNP mutation in the gene At1g15410
TCCATTGGATCAACACACTTCT	Reverse primer to verify presence of a SNP mutation in the gene At1g15410
GGCCTTGCTCTTGGTTACAA	Forward primer to verify presence of a SNP mutation in the gene At1g15690
CGAAGAAGGGAGCAAAGACA	Reverse primer to verify presence of a SNP mutation in the gene At1g15690
TGAATCCGGTTCATAAGAAAA	Forward primer to verify presence of a SNP mutation in the gene At1g17180
TTCCGAAACAAGGAAGAAACA	Reverse primer to verify presence of a SNP mutation in the gene At1g17180
CTAGCTTGGGCGTATTCTCG	Forward primer to verify presence of a SNP mutation in the gene At1g13570
GCGGAACAAGGAGACAAAAA	Reverse primer to verify presence of a SNP mutation in the gene At1g13570
GAGATCTTGGGTTGTGAATCG	Forward primer to verify presence of a SNP mutation in the gene At1g16110
CAAGCACCCTGTTCATGCT	Reverse primer to verify presence of a SNP mutation in the gene At1g16110
TGATGATCTTGGCGGCGTCT	Forward primer to verify presence of a SNP mutation in the gene At1g14120
AATCTATCTTTGGGACGTCACTT	Reverse primer to verify presence of a SNP mutation in the gene At1g14120
Primers looking for *sfr9* mutations	
TGGTGGTTGGTGGTCTAGGT	Forward primer to verify presence of a SNP mutation in the gene At5g62680
AGAGGATGGTGGAGATGTCG	Reverse primer to verify presence of a SNP mutation in the gene At5g62680

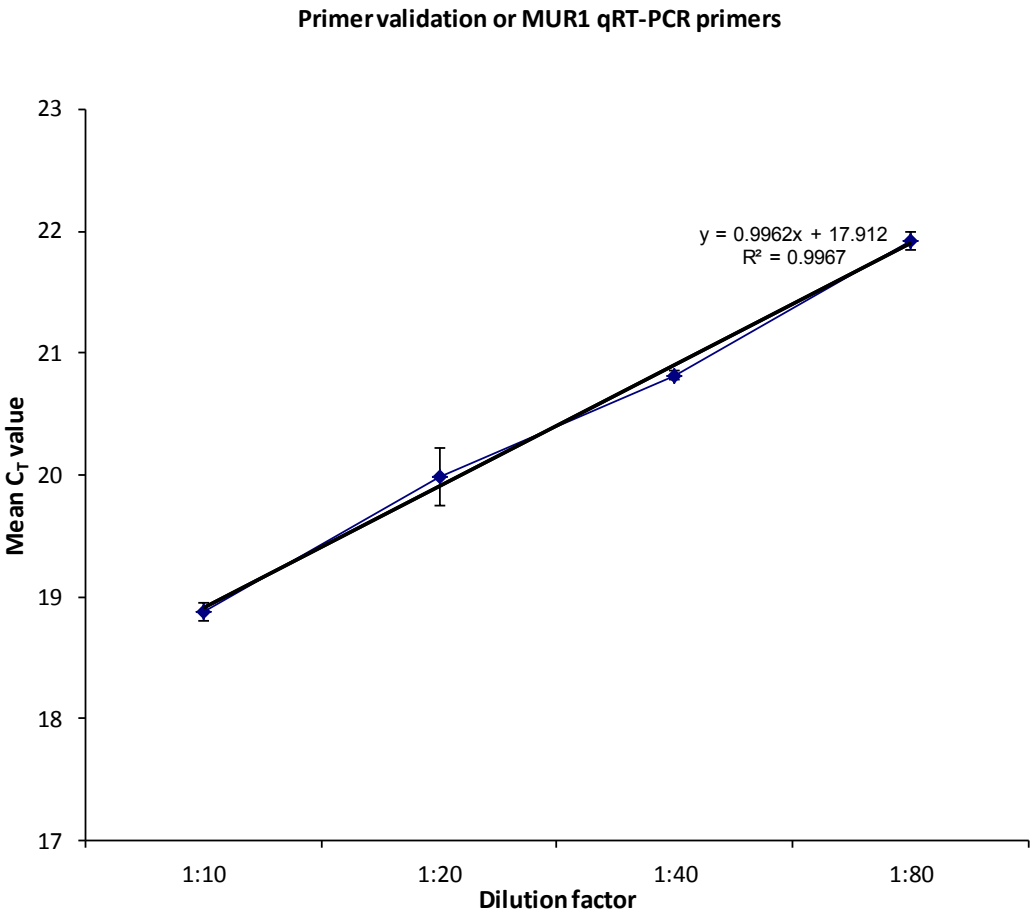
Sequence	Function
Primers for genotyping of T-DNA insertional mutants	
ATCTTCCTCAGCTTTCTTCCG	Right primer for genotyping of insertional mutants SALK_074693C and SALK_124555C
GCTGATTCACCTGGGTCATAG	Left primer for genotyping of insertional mutants SALK_074693C and SALK_124555C
TTGTTTCGGTGAAATTTCTGG	Right primer for genotyping of insertional mutant SALK_132810C
CCATCCCAAGCATCATAAAAC	Left primer for genotyping of insertional mutant SALK_132810C
TTTTCGTGCGAGAATTGAATC	Right primer for genotyping of insertional mutant SALK_150964
ATGACAAGGCGCTACTACTGC	Left primer for genotyping of insertional mutant SALK_150964
ACAATGGCGTCAGAGAACAAC	Right primer for genotyping of insertional mutant SALK_027379
AGCATCCATGTATCCAGCATC	Left primer for genotyping of insertional mutant SALK_027379
TTCCTTCTCGGAAAAGGCTAC	Right primer for genotyping of insertional mutant SALK_027387
TCATCCTCAACTCATTGGAGG	Left primer for genotyping of insertional mutant SALK_027387
AGCATCCATGTATCCAGCATC	Right primer for genotyping of insertional mutant SALK_057153
ACAATGGCGTCAGAGAACAAC	Left primer for genotyping of insertional mutant SALK_057153
GTGCGTTCTTCACTGAGCTTC	Right primer for genotyping of insertional mutant SALK_020645C
GGCTTTAAAATTCGGTGGATC	Left primer for genotyping of insertional mutant SALK_020654C
TAACAGAGCCACCGTATACGG	Right primer for genotyping of insertional mutant SALK_052178C
AACACGAAAACGTTGGTTGAC	Left primer for genotyping of insertional mutant SALK_052178C
ATTTTGCCGATTTCGGAAC	SALK LBb 1.3 (Left Border primer)
TCATTGTTGCATTCAGGTCTG	Forward primer for genotyping of insertional mutant GK596C07
GAGCATACCAAGAGCAGCAAC	Reverse primer for genotyping of insertional mutant GK596C07
GACAGACTGCCTAGCATTTGAGT	GABI-Kat Left Border primer

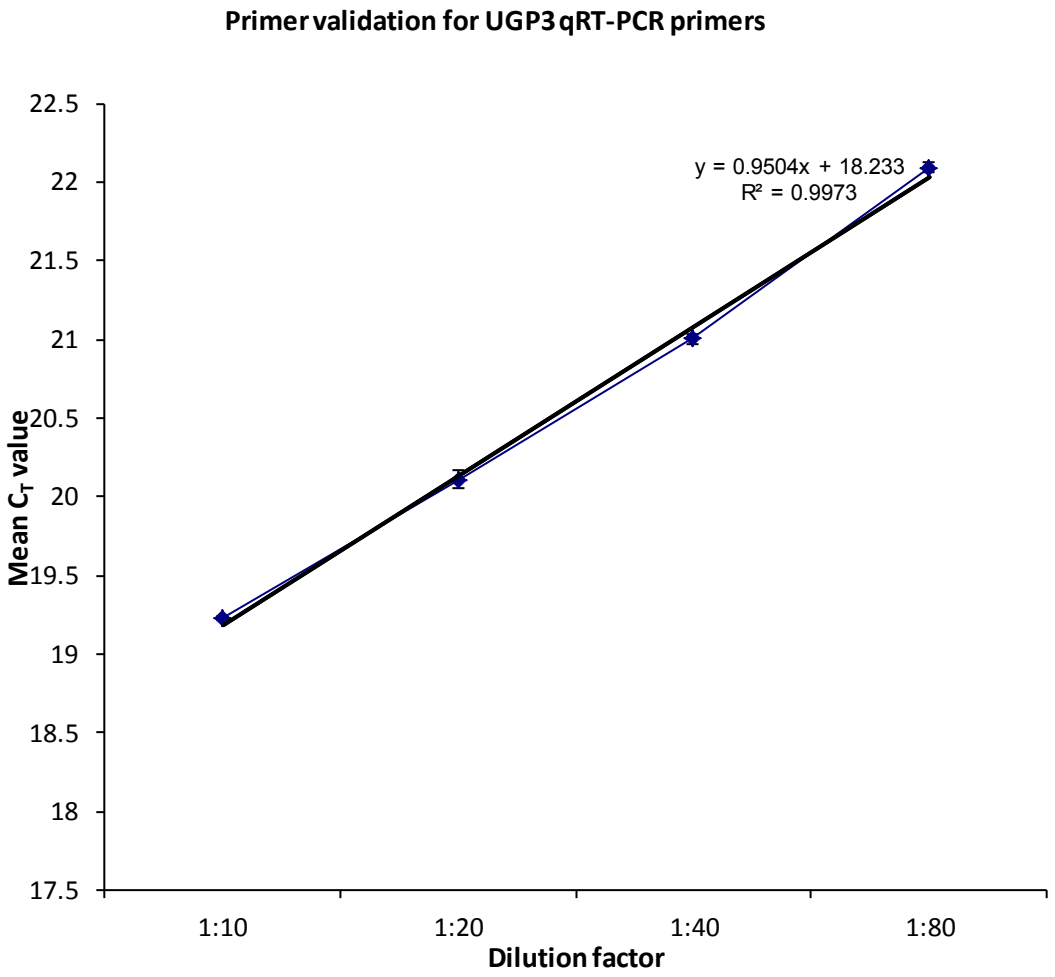
Sequence	Function
Primers for qRT-PCR (continued)	
CTGAAATCCCAGATCCGAAA	Forward primer to measure transcript levels of *MUR1* in Col-0
ATCTTCCTCGGTTCAACGAC	Reverse primer to measure transcript levels of *MUR1* in Col-0
AGAGCTGAAGGAGCGAGGAG	Forward primer to measure transcript levels of UGP3 in Col-0 and SALK_020654C
ACCATGGCTAATCCTCAAGC	Reverse primer to measure transcript levels of UGP3 in Col-0 and SALK_020654C
GAAAGTCGTTTATAGAGGCTGGA	Forward primer to measure transcript levels of *GTR2* in Col-0 and SALK_052178C
GTGTTCCAATGATCCCAAGC	Reverse primer to measure transcript levels of *GTR2* in Col-0 and SALK_052178C

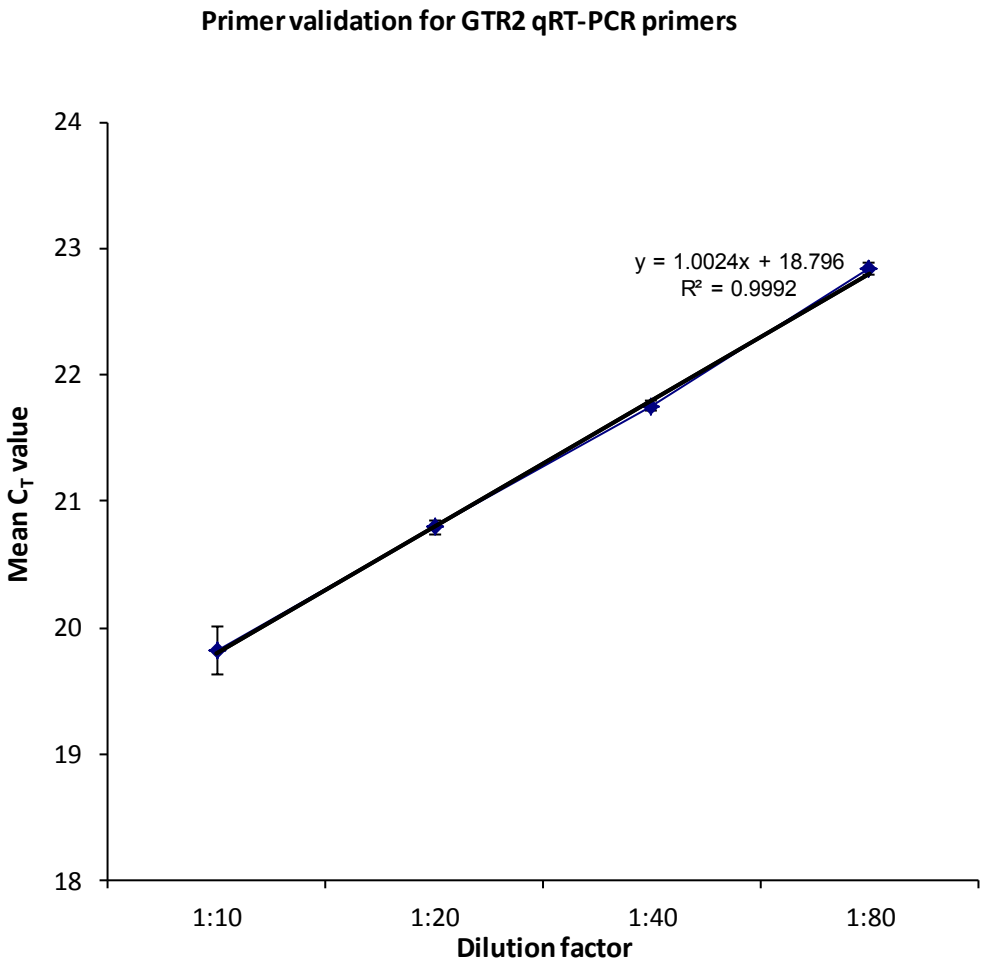
5.2. Primer verification graphs




Primer validation for At1g15410 qRT-PCR primers
y = 1.0004x + 21.162
R² = 0.9894
Mean C_T value
26
25
24
23
22
21
20
1:10
1:20
1:40
1:80
Dilution factor


Primer validation or MUR1 qRT-PCR primers
y = 0.9962x + 17.912
R² = 0.9967
Mean C_T value
23
22
21
20
19
18
17
1:10
1:20
1:40
1:80
Dilution factor


Primer validation for UGP3 qRT-PCR primers
y = 0.9504x + 18.233
R² = 0.9973
Mean C_T value
22.5
22
21.5
21
20.5
20
19.5
19
18.5
18
17.5
1:10
1:20
1:40
1:80
Dilution factor


Primer validation for GTR2 qRT-PCR primers
y = 1.0024x + 18.796
R² = 0.9992
Mean C_T value
24
23
22
21
20
19
18
1:10
1:20
1:40
1:80
Dilution factor

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